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Hyper Stable Variants of FGF-1-FGF-2 Dimer

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Hyper Stable Variants of FGF-1-FGF-2 Dimer

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Abstract

Fibroblast Growth Factors (FGFs), including FGF-1 and FGF-2, are proteins that play a crucial role in cell proliferation, cell differentiation, cell migration, and tissue repair.[1] FGF-1 and FGF-2 are useful in accelerating the healing process in the human body; however, these proteins are naturally thermally unstable, resulting in a relatively low half-life *in vivo*.[1,8] In efforts to improve the stability of this protein, FGF-1 and FGF-2 proteins are engineered by combining the amino acid sequences of the two proteins to form a heterodimer and obtain novel properties. These two FGF variants are chosen for their specific wound healing capabilities. FGF-1 non-selectively binds to all the known fibroblast growth factor receptors (FGFRs) and FGF-2 is strongly correlated with angiogenesis in a variety of bodily tissues, including muscle, adipose, bone, and tooth.[8] The heterodimer also contains two mutations within the FGF-1 portion of the dimer, R136E and K126N. These mutations were induced to negate the overwhelming positive charge present in the heparin binding pocket (HBP) of FGF-1 and were found to increase the overall stability of the protein, increase cell proliferation activity, and decrease heparin binding affinity.[10] The goal of this research project is to evaluate the dimer double mutant and compare its structure and function to that of the wild type FGF-1 and FGF-2 heterodimer. The heterodimer double mutant is found to have greater stability than the dimer wild type by having a greater trypsin resistance than the dimer wild type, as well as increased thermal stability both with and without the presence of stabilizing heparin. The continued investigation will provide valuable insight into the concept of FGF protein stability, and information regarding FGF dependence on heparin. This would enhance the knowledge pertinent to the possibility of novel wound healing agents comprised of FGFs in modern medicine.

Introduction

FGF Family

Fibroblast Growth Factors (FGFs) are a superfamily of cell signaling proteins found in a variety of organisms, including *homo sapiens*, that mediate and participate in various biological pathways relating to tissue repair and regeneration. These processes include cell proliferation, differentiation, and angiogenesis.[1] The mammalian FGF family contains 22 protein types. 18 of these proteins function as ligands, and the remaining four are fibroblast growth factor receptors (FGFRs), a classification of receptor tyrosine kinases that are anchored in the cell membrane.[2] The FGF proteins that are found in mammalian organisms include FGFs 1-10 and FGFs 16-23.[2] Mammalian FGF is further grouped into 6 subfamilies by additional similarities in structural and functional homology that are not entirely shared by other members of the superfamily.[2] The molecular mass of proteins in these subfamilies varies from 17 to 34 kDa.[1] The majority of FGF proteins have a shared internal structure comprised of six identical amino acid sequences and 28 highly conserved sequences, resulting in a 13-71% amino acid congruence.[4,21] A defining feature of the FGF family is the binding of heparin. Interaction between FGF and heparin, a glycosaminoglycan, functions to stabilize the protein in general as well as forming an FGF-heparin complex that has a higher FGFR binding affinity .[4] The resultant binding of an FGF protein to their respective FGFRs mitigates these biochemical processes by activating the receptor to transmit the signal to initiate the downstream signaling pathways.[1]

FGFR and Cell Signaling

Fibroblast growth factor receptors are a classification of four proteins among the 22 total mammalian FGF proteins and are a type of receptor tyrosine kinase (RTK). The structure of

these receptors includes a site for ligand binding, located extracellularly, in addition to the transmembrane portion of the receptor, and the intracellular tyrosine kinase.[1] The extracellular portion of receptor is known to possess two or three domains with structures similar to an immunoglobulin, or antibody, a segment of acids known as the "acid box", and a heparin binding area.[1] The first domain (D1) is associated with autoinhibition of the receptor, and binding specificity is associated with D2 and D3.[2] Three of the four FGFRs (FGFR1-FGFR3) have been found to have isoforms of the same receptor with a varied binding specificity and tissue specificity, as a result of alternative splicing occurring in domain 3.[1,21]

Once the FGF ligand binds to the extracellular portion of the FGFR and the receptor is activated, dimerization of the receptor begins.[2,21] This activation allows the cytoplasmic tyrosine kinase portion of the receptor to initiate autophosphorylation and consequently begin to transmit the cellular signal through the remainder of the cascade through additional transphosphorylation or by other cell signaling messengers.[3]

FGF-1 and FGF-2

The FGF-1 subfamily includes the acidic fibroblast growth factor, or FGF-1, and basic fibroblast growth factor, or FGF-2. The proteins share 55% of their structural identity as well as both proteins lacking classical peptide sequence signal for secretion, unlike the other members of the superfamily.[3]

Human FGF-1 is a protein comprised of 155 amino acids with a molecular mass of approximately 16 kDa.[3,10] The secondary structure of FGF-1 is 12 beta-sheets organized into a β-trefoil.[10] Contained in the structure of FGF-1 is a heparin binding pocket (HBP). Residues 122-142 located at the C-terminal end of the protein are attributed to being the heparin binding site of

FGF-1.[13] FGF-1 is a mitogen strongly associated with various tissue and cell types, including the dermal layers, suggesting a strong propensity for cell proliferation, tissue regeneration, and general wound repair.[5,6] In addition, FGF-1 is notable for its ability to bind to all four of the tyrosine kinase receptors as well as all known splice variants.[1]

First isolated in 1988, the FGF-2 gene in humans codes not for one protein, but varying isoforms.[8] The secreted human FGF-2 isomer has a weight of 18 kDa and has a primary structure that is 154 amino acids in length.[8] The secondary structure of FGF-2 also consists of 12 β-sheets. Evidence of angiogenic properties have been observed in a variety of human tissues, including adipose, osseous, nervous, and dermal tissue.[1,7]

Heparin and FGF-Heparin Interaction

Heparin binding is a universal trait in the mammalian FGF family. Heparin is a negatively charged, sulfated polysaccharide, with a molecular weight of ~15 kDa.[18] Heparin is comprised of repeating glucosamine and iduronic acid units, synthesized by the human body in a non-template manner, meaning the final structure can be altered during biosynthesis to produce a heparin molecule with the necessary binding capabilities for a specific physiological process.[18] The diversity in structure for heparin provides reasoning as to its biodiversity in mediating such a vast array of biological processes by binding with the protein involved in the process. The FGF protein specifically has a high binding affinity for heparin due to the strong electronegativity heparin possesses. Each of the sulfonated polysaccharide groups that are present in the structure of heparin contributes to the overall negative charge of the molecule, with the average heparin disaccharide containing roughly 2.7 sulfate groups.[18] Heparin is produced naturally by the human body but is also commercially produced by harvesting the heparin from bovine and porcine intestine tissue.[8]

Heparin or heparan sulphate proteoglycans (HSPG) are thought to play a vital role in the formation of the active FGF protein and receptor complexes necessary to transmit cellular signals. It is thought to be essential for two main reasons: to provide stability to FGF, and aid in denaturation prevention, as well as facilitate the activation of FGFR through FGF binding.

Heparin Effect on FGF-1 Stability

FGF-1 possesses an innate instability due to the abundance of positive charges located in a condensed region of the protein.[8,13] Positively charged amino acids within the heparin binding pocket (HBP) are located beside other positively charged acids creating an electrochemical imbalance owing to the repellent nature of like-charges in proximity.[3] When the repulsion takes place, the HBP further opens and becomes more susceptible to the action of proteases. To counteract this instability, negatively charged heparin binds to FGF-1 at a positively charged acid sequence, termed the heparin binding pocket HBP, that is located at the carboxy terminus of the polypeptide chain.[3] The resulting heparin-FGF complex is less susceptible to the denaturation due to proteases, heat, or acid.[3, 12]

Heparin Effect on FGF-FGFR Stability

Heparin or HSPG binds to both FGF and FGFR to promote FGF-FGFR binding affinity and stabilize the bond between the protein and the corresponding receptor.[2] Studies have shown that in some cases, the FGF-FGFR complex has formed in the absence of heparin and allowed for a partial level of activation, but the presence of a heparin molecule increases both stability of the complex and degree to which the receptor is activated.[21]

FGF Instability

Although heparin aids in the stability of the FGF, the protein is still susceptible to degradation within the wound healing process itself. Injury repair occurs in a regulated process that proceeds in three overlapping steps: blood clotting, tissue regeneration, and tissue remodeling.[11] Once a wound has been inflicted on the skin, the body initiates the clotting process to inhibit blood loss. The blood clotting cascade utilizes the enzyme thrombin in the process of converting the proteoglycan initially present at a wound site, fibrinogen, to a blood clot containing the mesh-like protein, fibrin.[10] After clotting, the FGF mediated process of tissue regeneration begins.[4] Although thrombin is an essential enzyme in the clotting cascade, it is also a protease that has been observed to denature FGF-1 by cleaving at R136, even with the additional stabilization effects of heparin.[13]

Thrombin, however, is not the only protease that has been found to denature FGF-1 and FGF-2.[8] Trypsin and chymotrypsin are two serine proteases found in the human body that are known to cleave and denature FGF. Trypsin cleaves the peptide bond on the c-terminal end of lysine and on the c-terminal end of arginine. Chymotrypsin is known to cleave on the c-terminal end of aromatic amino acids, such as tyrosine, phenylalanine, and tryptophan. Experiments which analyze the extent of protein degradation after varying degrees of exposure to proteases are a common characterization method in modern protein research.

Not only are FGF-1 and FGF-2 susceptible to proteolytic cleavage, but they also have a relatively low thermal stability, resulting in a short half-life both *in vitro* and *in vivo*.[8, 9] In the presence of heparin, FGF-1 half-life Is 26 hours, and in the absence of heparin, FGF-1 has a half-life of only 15 minutes.[22] The wild-type FGF-2 has been found to have a half-life of only 7.6 hours in the human body and an *in vitro* half-life of 10 hours at 37˚C.[2,8] At human physiological

temperature approximately half of the proteins' structures are unfolded, and therefore rendered inactive.[12] In addition, these denaturation effects are still often observed even in the presence of stabilizing heparin.[12]

Therapeutic Potential of FGF-1 and FGF-2

Although possessing an inherent instability, FGF has been studied extensively in medical literature and found to have impressive therapeutic potential. Since its first isolation in 1984, FGF-1 has been found to be effective in wound healing and tissue reparation. In 1994, FGF-1 was used to accelerate wound healing in diabetic mice by increasing cell proliferation of epithelial cells and collagen.[7] Nearly a decade later, in 2005, the first FGF-1 drug was approved by the Chinese FDA, and in 2006 an FGF product for diabetic ulcers was available to consumers for purchase.[7]

Numerous studies have shown that application of FGF-2 also has the potential to improve the healing process for patients. In one controlled trial, an aqueous solution of FGF-2 was applied topically to pressure wounds, and after the completion of the study, the patients had an increased number of capillaries and over 70% of wound closure.[25] FGF-2 was also utilized in a 2013 study of chronic wound patients in Kyoto, Japan. 16 out of the 17 patients who were treated with collagen/gelatin sponge (CGS) impregnated with FGF-2 experienced substantial wound bed improvement in lesions that were not expected to heal with conventional methods.[23]

It is evident that these proteins have been utilized in therapeutics and have immense potential for use in many more novel wound treatments. However, one consistent problem, inherent instability, reduces their practical use and limits their efficacy. Once FGF is administered to wound sites, the biological function of the protein is rapidly lost and the FGF

compound must be constantly and frequently readministered to produce the intended effect.[8] Further stabilization of these proteins would progress the production of commercially available FGF comprised pharmaceuticals in a variety of medical arenas and would benefit many demographics. Currently, there are glaring healthcare issues facing people groups both globally and domestically that would be remedied by development of stable FGF therapeutics.

Globally, underserved communities in developing countries need pharmaceuticals with revolutionary wound healing properties, like FGF. A stable FGF compound would greatly benefit these communities because mortality as a result of infection is disproportionately high in developing countries; the rate of mortality from infections incurred post-surgical procedures is 10 times greater than that of developed countries.[16] These surgical site infections (SSI) include a wide spectrum that ranges from superficial dermal infection to life-threatening septicemia, yet every SSI requiring medical diagnosis and treatment continues to place a financial burden on the healthcare system of these countries and acts as a hindrance to the countries' continued development.[16] A decreased duration of wound healing directly correlates with a decreased risk of infection.[27,28] If wound healing time was decreased by use a FGF pharmaceutical, the risk of infection, and therefore, this augmented rate of mortality would also decrease. In addition, these developing countries would be able to redirect the economic resources currently focused on the additional and recurring treatments of SSI patients towards pursuing continued community progression towards economic growth. This would result in a better quality of life for surgical patients as well as benefiting these countries as a whole.

The need for stable FGF based pharmaceuticals is also evident here in the Unites States. It is estimated that by 2035, the world census will have more elderly adults aged 65 years or older, than the population of children aged 18 years old and younger.[20] Considering one out of

every three adults over the age of 65 has diabetes, this statistic is a reason for concern.[19] Another alarming statistic is the national health spending on diabetes, which is projected to grow to $825 billion by 2035 and $845 billion in 2040.[7] Diabetes is known to cause neuropathy and any wounds that are incurred on the extremities may be difficult to locate and therefore treat, and as a result, are highly susceptible to infection. These infections may result in life-altering amputations, or potential death. According to the American Diabetes association, diabetic adults are the demographic presenting the greatest risk for infection-related mortality.[15] Even if these wounds are located and provided with adequate treatment, diabetic wounds do not readily heal and the delayed tissue repair process is not only an additional risk-factor for infection, but often results in recurrence of lesions at previous wound sites and is physically painful and a financial burden for the diabetic.[7] Increased wound healing rate and efficiency would provide diabetics with the necessary healthcare to live more comfortably, prevent fatalities, and decrease overall spending due to the rising cost of chronic wound treatment.

The goal of this study is to engineer an FGF variant with increased stability. This study investigates the stability of a novel FGF-1-FGF-2 heterodimer as well as this heterodimer with two mutations present on the FGF-1 portion of the dimer, R136E and K126N. The FGF-1-FGF-2 heterodimer is not a naturally occurring member of the fibroblast growth factor family. The dimer was synthesized by the Kumar group by linking the primary structure of FGF-1 to that of FGF-2 by means of a 12-residue glycine linker. The amino acid sequence of the FGF-1-FGF-2 heterodimer is:

MFNLPPGNYKKPKLLYSSNGGHFLRILPDGTVDGTRDRSDQHIQLQLSAESVGEV
YIKSTETGQYLCMDTDGLLYGSQTPNEECLFLERLEENHYNTYISKKHAEKNWFVGL**N**K
NGSAKRGP**E**THYGQKAILFLALPVSSDGGGGGGGGGGGGGGMAAGSITTLPALPEDGG

SGAFPPGHFKDPKRLYCKNGGFFLRIHPDGRVDGVREKSDPHIKLQLQAEERGVVSIKG

VCANRYLAMKEDGRLLASKCVTDECFFFEKLESNNYNTYRSRKYTSWYVALKRTGQY

KLGSKTGPGQKAILFLPMSAKS

where the bolded letters represent the mutations present in the amino acid sequence of the heterodimer molecule.

The resulting heterodimer is 305 amino acids in length with a molecular weight of 33.9 kDa. By combining both proteins, the dimer is expected to exhibit the advantageous properties of both FGF variants within a single polypeptide. The most advantageous characteristics of FGF-1 include the non-selective FGFR binding it exhibits as well as the protein's cell proliferation properties.[2] The most advantageous characteristic of FGF-2 is the protein's association with angiogenesis.[2] Both cell proliferation and angiogenesis are necessary for efficient wound healing, and the combination of both properties in this heterodimer is expected to contribute to more effective and rapid wound healing.

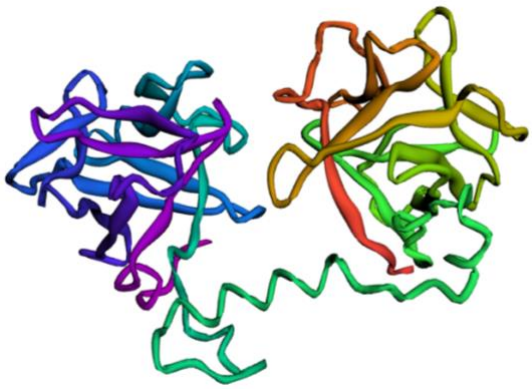

Figure 4. *FGF-1-FGF-2 Heterodimer Structure: FGF-1 (right) and FGF-2 (left) Linked via 12-Residue Glycine Linker*

The two mutations present in the FGF-1 portion of the heterodimer are chosen due to increased stability observed as a result of the mutations in previous studies conducted by the Kumar group. These two mutations are part of a series of five total mutations that will be induced on the dimer in future studies conducted by the Kumar group: R136E, K126N, Q54P, S61L, and H107S.

This R136E / K126N dimer double mutant possesses a mutation in which arginine-136 is converted into glutamic acid to prevent protease cleavage that occurs at the C-terminus end of arginine. Trypsin is a protease found to cleave proteins on the c-terminal end of lysine, K, and arginine, R. By introducing this mutation, trypsin digestion is prevented at this site. In addition, in a previous study published by the Kumar group, it was found that in the wild type FGF-1 protein, thrombin cleaves at the c-terminal side of R136. Typically, the thrombin digestion pattern is that the protease cleaves polypeptides at the c-terminal end of arginine when an L-V-P-R-G-S amino acid sequence is present, however, this amino acid sequence is not present in FGF-1, indicating that a secondary cleavage site must be present. The mutation of arginine to glutamic acid mutation at amino acid 136, was observed to consequently prevent the protein from experiencing any degree of degradation from thrombin exposure.[13] Although the mutation prevents thrombin cleavage, the thermal stability of the protein only marginally increased.[13] A variety of neutral amino acid substitutions were attempted, including G, L, Q, and K, however, E was discovered to yield the most stable mutant.[13] Overall, the R136E mutation was found to cause the protein to exhibit greater stability and cell proliferation activity than the wild type, even with a reduced heparin binding affinity, a surprising observation.[13]

The dimer mutant being investigated also possesses a mutation in which lysine-126 is converted into asparagine. This mutation was also investigated in the same Kumar group study.

The rationale for designing the K126N mutant was that the charge nullification of a positive acid located within the HBP should decrease repulsion, tighten the heparin binding pocket of FGF-1, and increase protein stability. Although N and Q are both uncharged amino acids, it can be assumed that N was chosen as the amino acid replacement for K, because mutation to Q has been found to cause aggregation of the protein, preventing the ability to easily perform accurate characterization studies. In addition, repeating sequences of glutamine can have potentially detrimental effects if a mutation causes the chain to expand beyond the threshold for disease.[24] When abnormally long polyQ expansions occur, the resulting aggregation of the protein is likely to contribute to the onset of a neurodegenerative disease. For example, when an abnormal polyQ expansion occurs as a result of a mutation in protein Htt, Huntington's disease usually results.[14] FGF-1 does have a polyglutamine tract, or a long sequence of consecutive glutamine molecules, and contributing an additional Q residue in place of K could cause instability and therefore, disease. The R136E / K126N double mutant variation of FGF-1 was found to have a marginally greater thermal stability and cell proliferation rate than the wild type FGF-1, yet again with evidence of decreased heparin affinity.[13]

Classically, it has been assumed that heparin or HSPGs are necessary for the FGFs to activate the FGFRs and therefore initiate cell proliferation, however, these recent studies show evidence of decreased heparin dependency with maintenance of proliferation potential. This suggests that heparin may not be a vital stabilizing component of FGF signaling cascades as historically assumed. This is significant because heparin is not ideal as a stabilizing factor for FGF due to its anticoagulant properties as well as safety and ethical concerns due to the porcine and bovine derivation of pharmaceutical grade heparin.[8] Overall, an FGF protein engineered to have greater thermal stability and proteolytic resistance, with complete or greatly reduced

heparin independence would be most ideal for future use in desperately needed FGF pharmaceuticals.

Methods

Cloning and Sequence Analysis:

E. Coli is a bacterium with a high yield for recombinant protein production, therefore the plasmids containing the DNA sequences encoding the mutant variant of FGF-1 and FGF-2 dimer are synthesized and transformed into the *Escherichia Coli (BL21 pLyss)*. The specific bacterial expression vector used is pET24a, chosen for its kanamycin resistance and restriction enzyme cloning capabilities.

Overexpression and Purification:

The FGF 1-FGF 2 *E. coli* glycerol stocks were cultured for a large-scale expression. 1 mL of glycerol stock was used to inoculate 200 mL of sterilized LB media and 200 μL of 100 mg/mL ampicillin. This culture was incubated for 12-16 hours in a New Brunswick Science Innova 4330 Refrigerated Incubator Shaker at 37℃. The next morning, 25 mL of the overnight culture was added to 8 flasks containing 500 mL of LB media and 500 μL of 100 mg/mL ampicillin. The cultures were then placed in the same shaker at 37℃ and allowed to incubate for 1-1.5 hours until the OD reached 0.6-0.8. 500 μL of IPTG was added once the OD reached the desired value and the cultures were allowed to grow for 4 hours in the shaker at 37℃.IPTG is a mimic of allolactose, it binds to the lac repressor. This binding induces the release of the lac repressor, which allows the transcription of genes in the lac operon, namely Beta-galactosidase, Beta-galactoside permease, and Beta-galactoside transacetylase. The transcription of these genes induces the expression of the protein. After this incubation period, the cells were harvested by

centrifuging the cultures at 6000 rpm for 25 minutes in Thermo Scientific Sorvall Lynx 6000 centrifuge. The supernatant was discarded, and the pellets of the samples were redissolved in a buffer and centrifuged again following the same procedure as before. The supernatant was once again discarded, and the final pellets were stored at -20℃ until purification.

For purification, the pellets were resuspended in 35 mL of buffer (10 mM phosphate buffer) and sonicated at 10 seconds on and 10 seconds off for 25 minutes with a Branson Sonifier 150 to lyse the bacterial cell wall and membrane. After sonication, the solution was centrifuged in a Thermo Scientific Sorvall Lynx 6000 centrifuge at 19000 rpm for 20 minutes after transfer to Oak Ridge tubes. The supernatant was then loaded onto a pre-equilibrated (with 10 mM phosphate buffer) heparin-Sepharose column and heterodimer protein was eluted with prepared phosphate buffer solutions of varying NaCl compositions, including 100 mM, 300 mM, 500 mM, 800 mM, 1250 mM, and 1500 mM solutions of NaCl to isolate the mutant dimer. The purity of the eluted fractions was analyzed via SDS-PAGE.

Fluorescence and Circular Dichroism Spectroscopy:

Circular dichroism and intrinsic fluorescence spectra from a Jasco J-1500 Spectrophotometer were obtained to determine the secondary and tertiary structural changes of the FGF 1-FGF 2 heterodimer. Circular dichroism (CD) is used to determine the secondary structural changes of a protein while fluorescence is used to determine the tertiary structural changes. 33 μM protein was added to 10 mM PB and loaded into a 1 cm path length quartz cuvette. To obtain the CD spectrum, the machine scanned the protein sample at a speed of 20 nm/min over the range of 190-250 nm at 25ºC. The CD data was acquired as an average of three scans. To obtain the intrinsic fluorescence spectrum, the excitation wavelength was set to 280

nm, and the emission spectra was set for a range of 300 to 450 nm. The fluorescence data sheds light on the tertiary folding of the protein based on the naturally fluorescent tryptophan and tyrosine residues, which have an emission wavelength of approximately 320-350 nm and 305-308 nm, respectively.

Differential Scanning Calorimetry (DSC):

DSC is a technique used to determine the melting point of the protein. The protein is compared to the 10 mM sodium phosphate reference buffer containing 100 mM NaCl over a temperature interval of 20˚C to 85˚C, in 5˚C increments, at a rate of 90˚C/hr. The temperature change over time plotted against the heat capacity allows for the determination of the melting point, the point at which 50% of the protein is denatured. The samples of FGF-1-FGF-2 wild-type and double mutant were analyzed separately in samples with heparin and without heparin.

Limited Thrombin Digestion:

Proteolytic thrombin digestion was also performed to determine the structural flexibility of the wild-type heterodimer and the R136E / K126N dimer double mutant. Thrombin recognizes the specific amino acid sequence LVPRGS and cleaves between the arginine (R) and glycine (G) residues. Thrombin cleavage was performed using 0.48 mg/mL of FGF 1-FGF 2 heterodimer proteins. The samples were incubated at 37º C for the following lengths of time: 2, 4, 6, 8, 10, 30, 45, and 60 minutes. TCA preparation was performed on each sample immediately after the incubation to stop the thrombin reaction. Two control samples, one of protein without trypsin, and one of trypsin without protein are also analyzed for 2 minutes at 37˚C. An SDS-PAGE analysis was then conducted, to characterize the trypsin cleavage patterns of the mutant protein. A densitometric scan was conducted on the resulting SDS-PAGE gel to quantitatively analyze

the degree of trypsin digestion. The trypsin digestion was performed with and without heparin present in the protein sample.

Results and Discussion

Large Scale Expression and Purification

The resultant purified fractions are analyzed by SDS-PAGE to verify the protein's presence and purity. Figure 5 shows the results of the purification of the wild-type heterodimer molecule, with the desired protein band highlighted. Figure 6 shows the purification results of the double mutant dimer. Since both protein variants are the same molecular mass, they exhibit the same mobility on the SDS-PAGE gel. Contaminations are present in the same sample as the desired protein, therefore further purification is required.

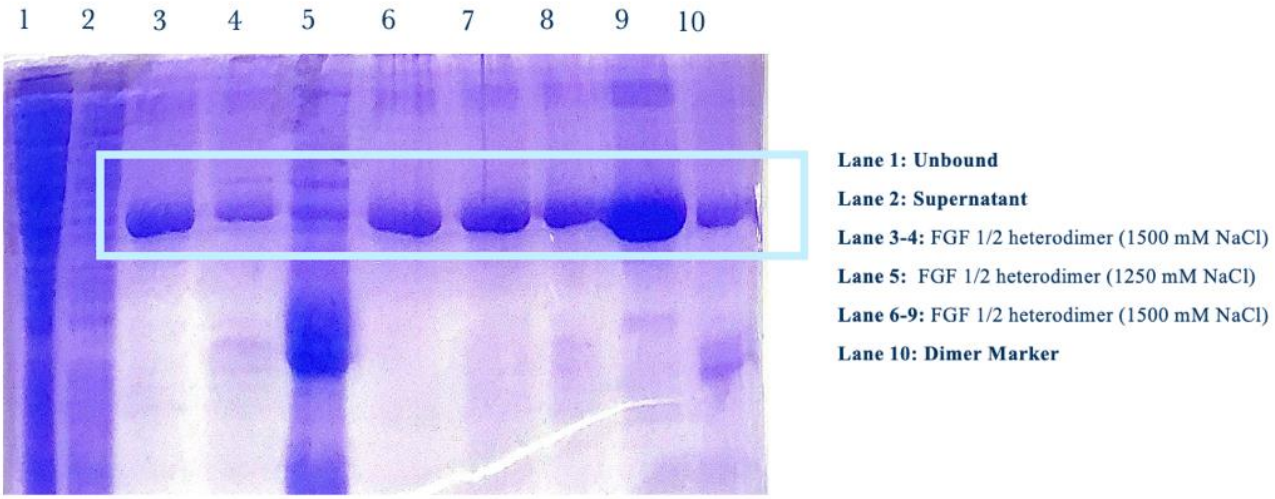

Figure 5. *SDS-Page analysis of FGF-1-FGF-2 Fractions Obtained from Heparin-Sepharose Column Chromatography.*

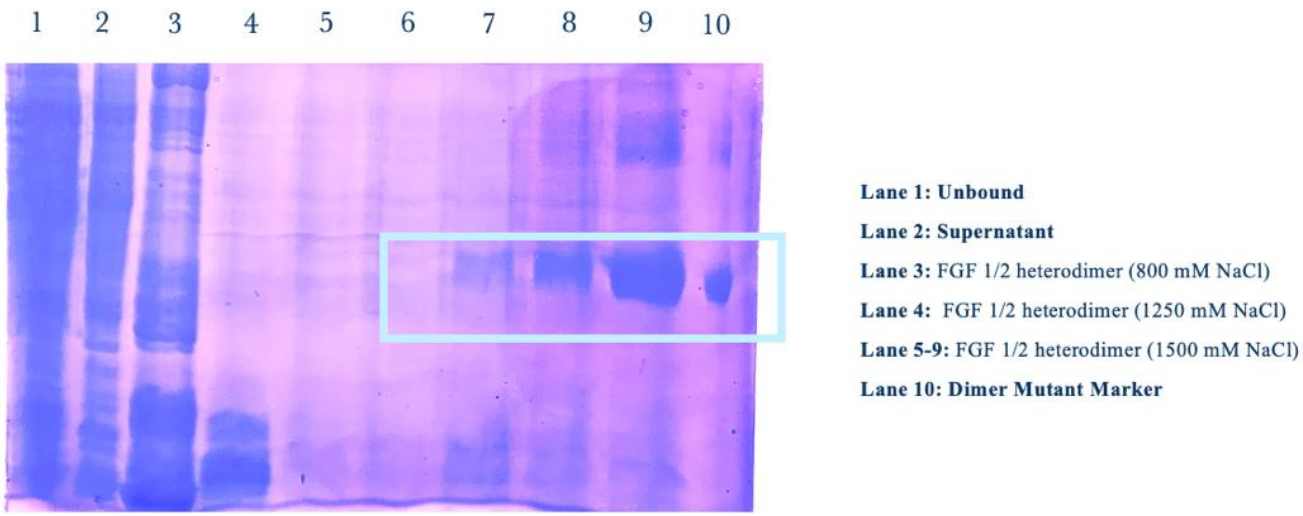

Figure 6. *SDS-Page Analysis of FGF-1-FGF-2 Double Mutant Fractions Obtained from Heparin-Sepharose Column Chromatography*

Structural Integrity

Circular Dichroism (CD) utilizes light to determine the secondary structure of a protein. In the resultant graphical data, different ellipticity peaks correspond to either α-helices, ß-sheets, or random coils in the secondary structure. Figure 7 shows the CD spectra of the FGF-1-FGF-2 wild-type protein, that of the R136E mutant, and the R136E / K126N double mutant. A positive peak is visible around 225 nm, and a negative peak is located around 205 nm. The positive peak indicates a ß-barrel structure, and the negative peak indicates the presence of antiparallel ß-sheets. As there is no significant change in the protein's CD spectrum after mutation, it can be suggested that the overall conformation of the protein is maintained after mutation.

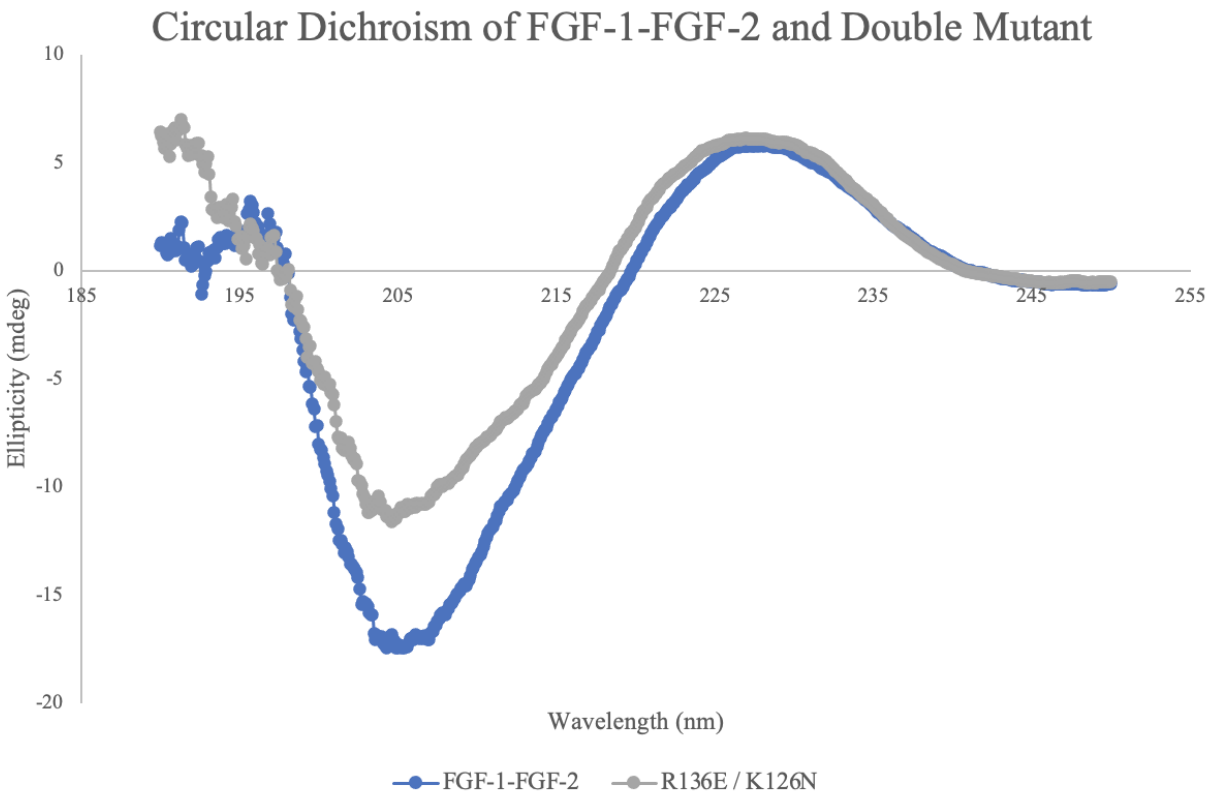

Figure 7. *CD Spectra of FGF-1-FGF-2 Dimer Wild-Type, R136E Mutant, and R136 / K126N Double Mutant*

Fluorescence Spectroscopy analyzes a sample using light to detect aromatic amino acids on the surface of a compound. Different intensity peaks correspond to either tryptophan, phenylalanine, or tyrosine. For the structure of FGF, tryptophan is present within the inner folds of the protein due to its hydrophobic nature. The only tryptophan present on the outer fold of FGF is not visible in the fluorescence spectrum because the indole ring of the tryptophan is quenched by the nitrogen-containing cyclic groups like proline, arginine, and lysine, which are close to tryptophan in the native state. If the protein denatures, the nitrogen-containing groups shift away from the tryptophan, the amino acid is then able to fluoresce, and the resulting peak appears on the graph. The peak present at approximately 308 signifies that tyrosine is present on

the outer fold of the analyzed protein samples and no tryptophan peak is present on the spectrum, indicating the structure of the protein has been maintained after mutation.

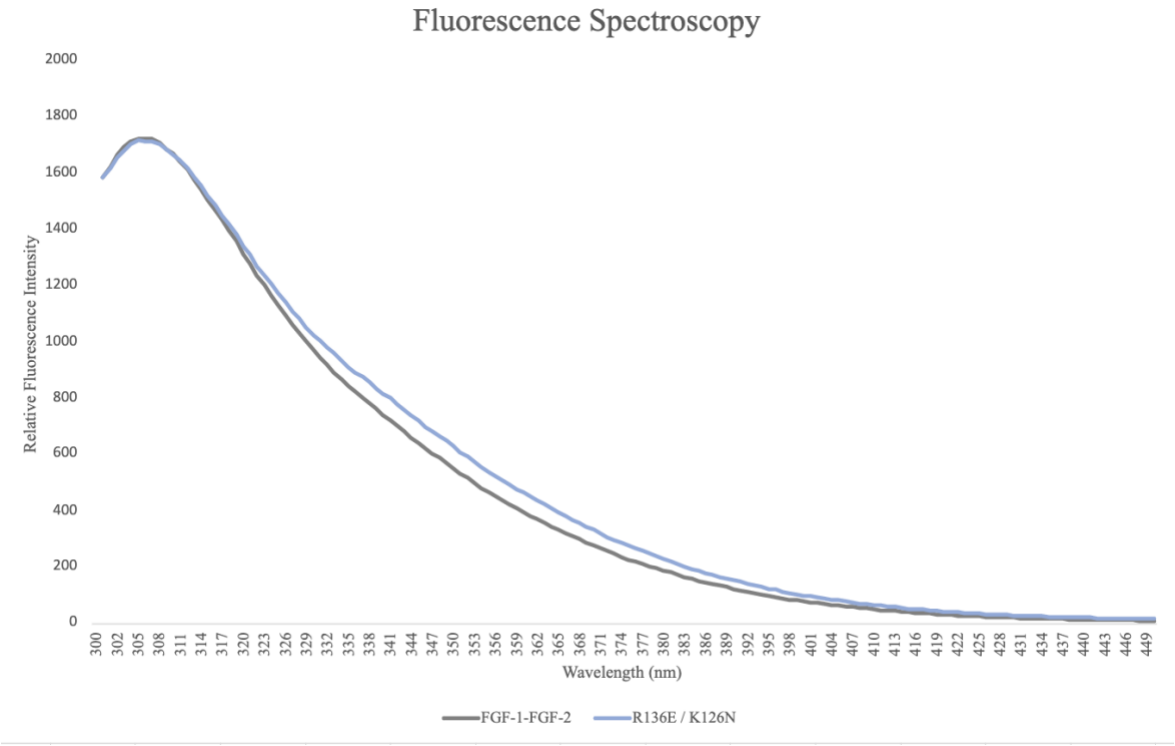

Figure 8. *Fluorescence Spectroscopy of FGF Heterodimer with and without R136E / K126 Mutations*

Thermal Stability

The wild-type FGF heterodimer was found to have a T_m of 51.43˚C, and a T_m of 56.32˚C when heparin was included in the sample. The R136E / K126N heterodimer was found to have a T_m of 52.82˚C without the presence of heparin, and a T_m of 66.56˚C when heparin was included in the sample.

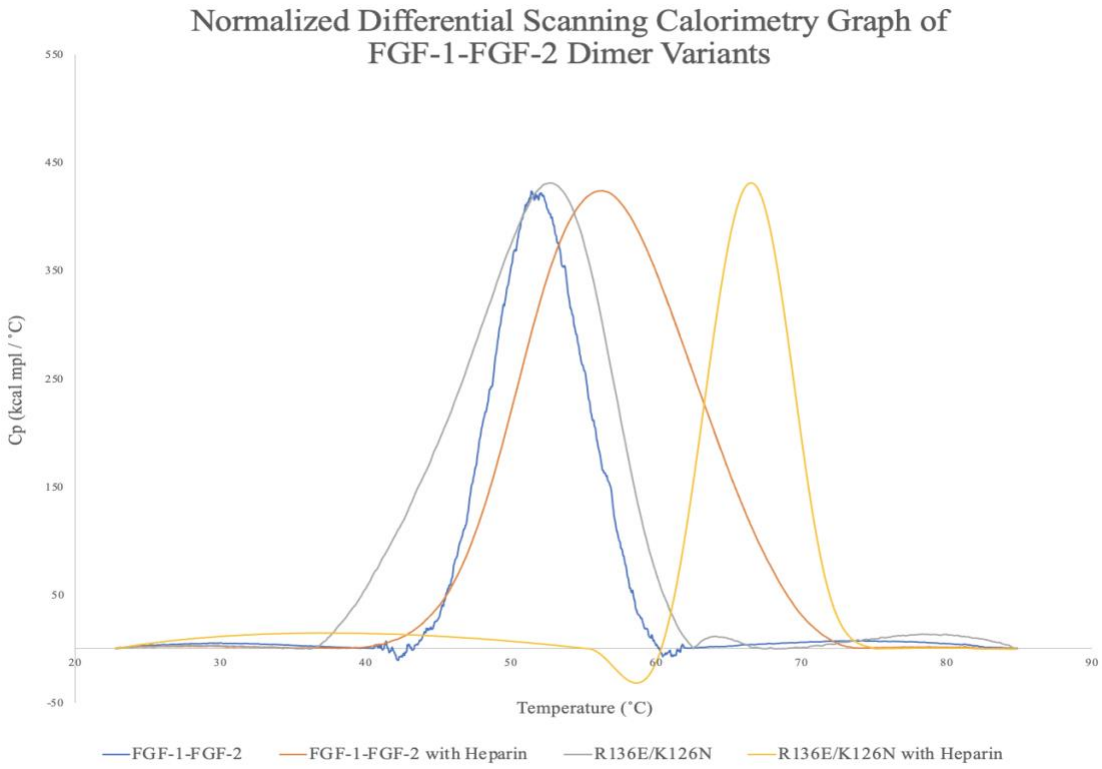

Figure 9. *Differential Scanning Calorimetry (DSC) of FGF-1-FGF-2 Variants*

The double mutant with and without heparin had a higher T_m than the respective wild-type dimer sample, indicating that a greater increase in thermal energy was required in these cases to reach the melting temperature. This indicates that the R136E / K126N double mutant is more thermally stable than the wild-type protein, and that heparin aids in increasing the stability of both FGF variants.

Interestingly, the FGF-1-FGF-2 heterodimer exhibits greater thermal stability than wild-type FGF-1. The melting temperature of FGF-1 according to a study conducted by the Kumar group is 41.5˚C, which is 10˚C lower than the melting temperature of the wild-type heterodimer without additional mutations.[10] The R136E/K126N double mutant of the FGF-1-FGF-2 heterodimer also exhibited a greater thermal stability than the FGF-1 R136E/K126N double

mutant investigated in the same study.

Trypsin Digestion

Trypsin is a protease that is found to cleave the peptide bond on the c-terminal side of any lysine and arginine present in the amino acid sequence of a protein. This cleavage results in a change of structure and denaturation of the protein. Since both R and K are present in a high concentration on the surface of the FGF protein, the trypsin digestion experiment is used to provide indication of the backbone flexibility of the FGF protein sample.

After exposure of trypsin to the protein for a controlled amount of time, an SDS-PAGE experiment was conducted to measure the level of protein degradation caused by the protease. The digestion activity can be qualitatively observed by the appearance of the gel's protein bands but quantified using densiometric scan. Trypsin digestion experiments were conducted with and without the presence of heparin, on both the heterodimer and the heterodimer with R136E / K126N mutations. The qualitative data from the time-dependent trypsin digestion is evident in resulting gels which are shown in Figures 10, 11, 12, and 13. A prominent protein band indicates the resistance of the protein to digestion, whereas the less intense protein band indicates a high degree of digestion of the protein. The qualitative data provide additional visual confirmation of the densitometric scan results that suggest the increased stability and proteolytic resistance as a result of introducing the R136E/K126N mutations on the heterodimer. The resulting densitometric data is presented in Figure 14.

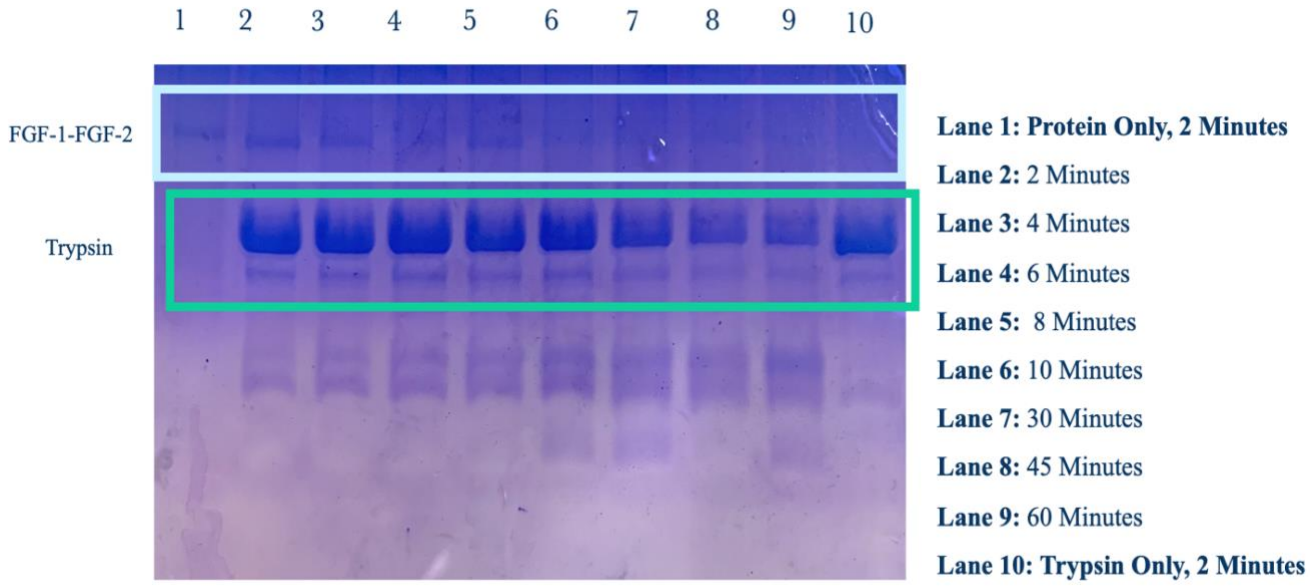

Figure 10. *Trypsin Digestion of FGF-1-FGF-2*

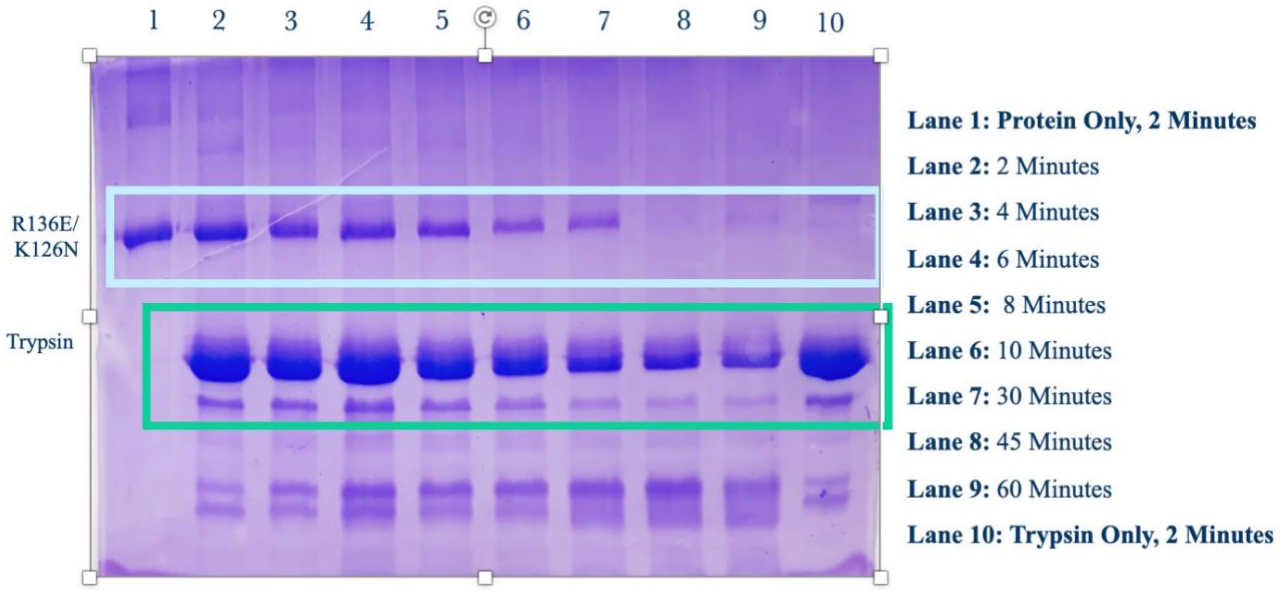

Figure 11. *Trypsin Digestion of FGF-1-FGF-2 Double Mutant*

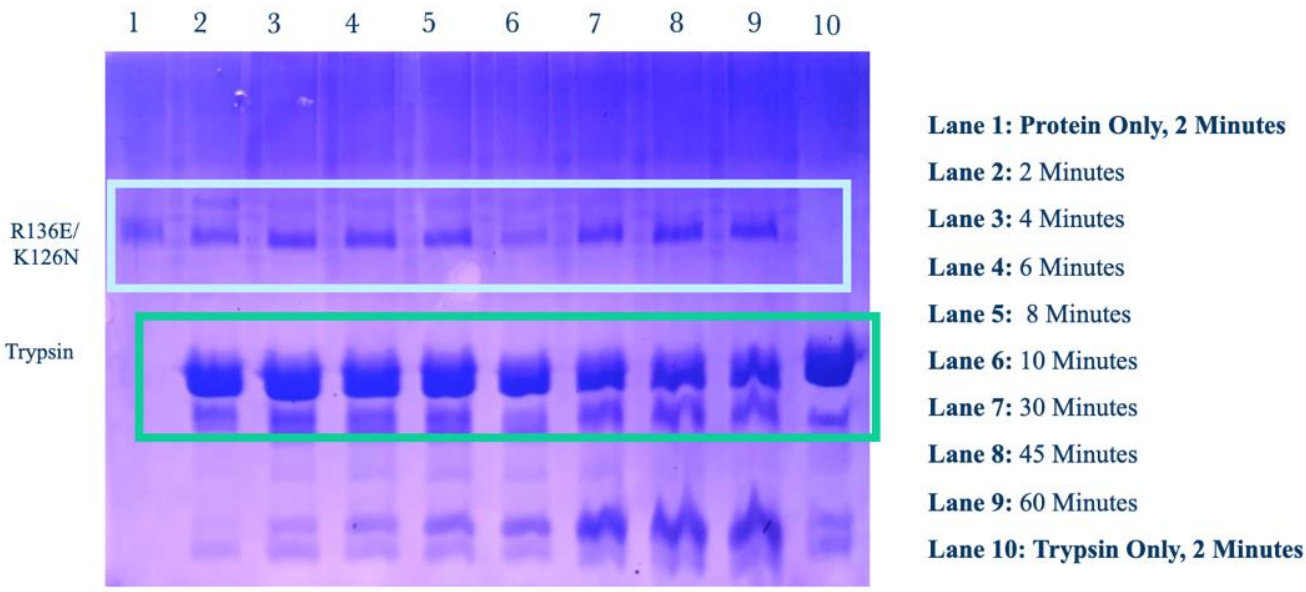

Figure 12. *Trypsin Digestion of FGF-1-FGF-2 Double Mutant in the Presence of Heparin*

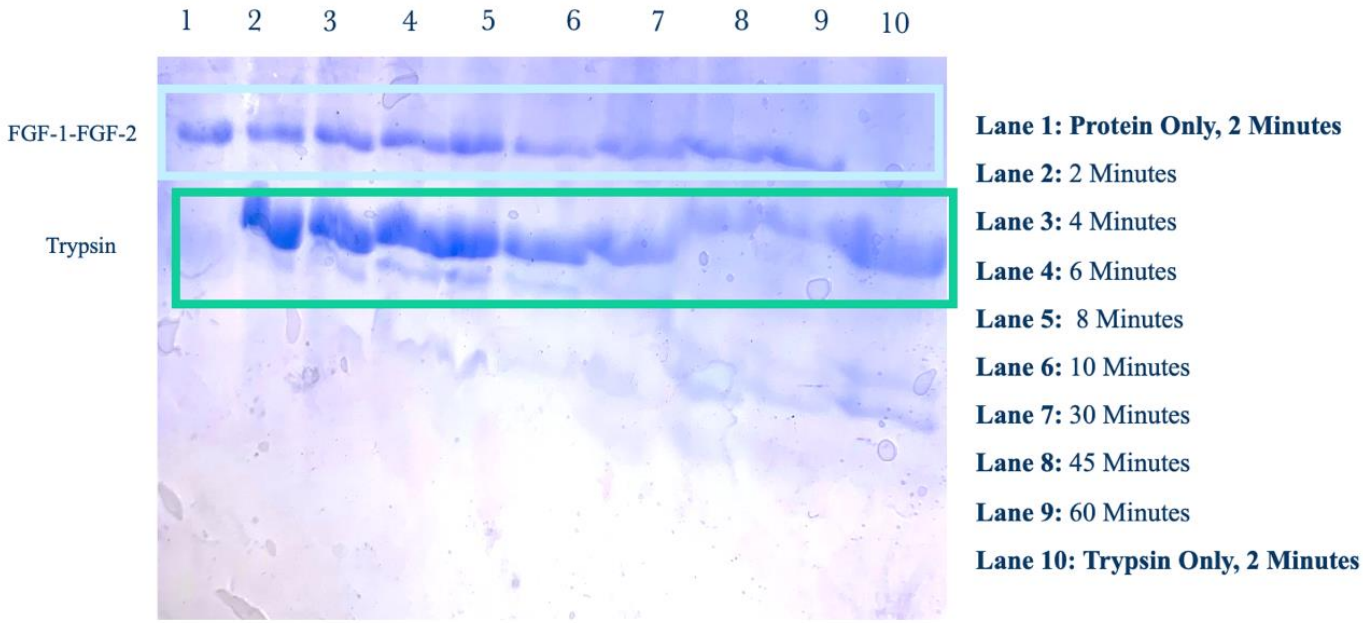

Figure 13. *Trypsin Digestion of FGF-1-FGF-2 in the Presence of Heparin*

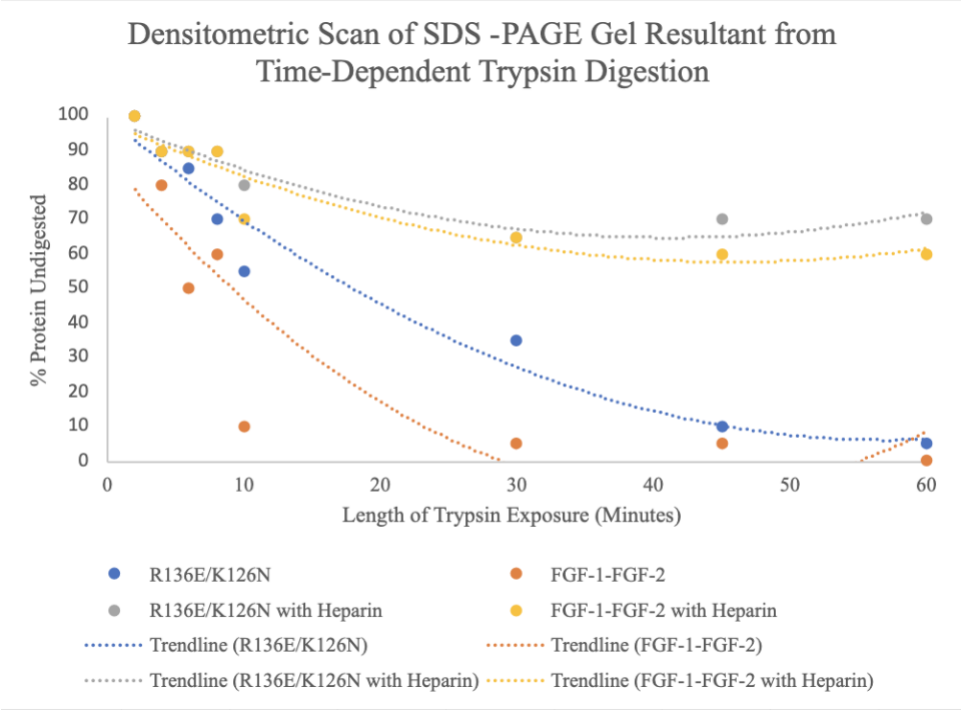

Figure 10. *Densiometric Scan of FGF-1-FGF-2 with and without R136E / K126N Mutations in the Presence and Absence of Heparin*

A greater density value signifies less trypsin digestion and more protein that has remained intact. In the trypsin digestion experiments with and without the addition of heparin, the heterodimer R136E/K126N double mutant is found to exhibit greater stability than the heterodimer, evident by the overall greater density percentages and higher trendline values. The addition of heparin to both protein samples increases trypsin resistance and therefore indicates reduction in the protein backbone flexibility.

When compared to previous studies conducted by the Kumar group, the heterodimer without mutation is once again found to demonstrate greater stability than the wild-type FGF-1 compound due to greater proteolytic resistance. After 15 minutes of trypsin exposure, 10% of the

FGF-1 protein was recorded to be undigested, in comparison to the 10% of undigested FGF-1-FGF-2 heterodimer protein remaining after 30 minutes of trypsin exposure.[13]

Conclusion

The FGF-1-FGF-2 heterodimer has greater thermal stability and trypsin resistance than the wild-type FGF-1 protein. The melting temperature of the heterodimer is 51.43 ˚C and the melting temperature of FGF-1 as discovered by a previous study performed by the Kumar group is 41.5˚C, which indicates a substantial increase in stability as a result of synthesizing FGF-1-FGF-2.[10] When the DSC FGF-1 double mutant investigated in the same study is compared to the FGF-1-FGF-2 double mutant, a higher melting temperature, and therefore thermal stability is observed in the dimer as well. Comparing the trypsin digestion results of wild-type FGF-1 and the heterodimer without mutations also demonstrates that the dimer has a greater trypsin resistance than FGF-1.

Comparing data obtained in this study and a prior study performed by a graduate student in the Kumar group provides evidence that suggests that the FGF-1-FGF-2 heterodimer exhibits greater stability than wild-type FGF-1, and therefore greater potential for use in pharmaceuticals. The double mutation of the heterodimer is shown to further increase the protein's stability without altering the conformation or structure. The fluorescence spectrum also indicates that the conformation of the protein is not compromised as a result of mutation. The circular dichroism results indicate that the secondary structure of the protein is maintained, and the ß-sheet barrel is not altered after mutation. The mutation of arginine to glutamic acid at amino acid 136 and the mutation of lysine to asparagine at amino acid 126 on FGF-1-FGF-2 is also found to result in a greater backbone flexibility and thermal stability than the wild-type heterodimer in both the

presence and absence of heparin. This is evident due to an increased trypsin resistance and higher melting temperature observed in the dimer mutant when compared to the unmutated dimer.

The mutations introduced in the dimer contributed a negative charge in the heparin binding pocket, which was assumed to likely decrease heparin binding affinity due to the electronegativity of the biomolecule and potential repulsion as a result. However, even after mutation of the heterodimer, FGF-heparin binding still occurs in the purification column as well as FGF is still shown to exhibit additional stability in the characterization experiments upon addition of heparin to a mutant FGF sample. For this reason, it is evident that heparin continues to bind to the FGF-1-FGF-2 heterodimer after the mutation is introduced.

Overall, the FGF-1-FGF-2 heterodimer is found to have greater stability than the wild-type FGF-1 protein, and the R136E/K126N double mutation introduced on the heterodimer results in even greater stability. Important insight on stability-increasing mutations has been discovered, as well as insight on FGF stability in the absence of stabilizing heparin. These discoveries provide valuable data that can be expected to aid in the design of an efficient FGF-based therapy for chronic wound care and infection prevention.

Future Perspectives

In the future, it would be beneficial to conduct heparin binding affinity experiments to gather additional evidence regarding the role of heparin binding in FGF cell signaling and overall functionality. In addition, cell proliferation assay experiments would need to be conducted to determine the bioactivity of the mutant protein and therefore give more in-depth insights as to the potential therapeutic benefits that the protein variants would possess.

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