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Characterization of FGF1/FGF2 Heterodimer with Mutation R136E

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Characterization of FGF1/FGF2 Heterodimer with Mutation R136E

An *Honors Thesis* submitted in partial fulfillment of the requirements for the degree of Honors Studies in Biology.

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Table of Contents

Acknowledgement *2*

Abstract *4*

Introduction *5*

Methods and Materials *11*

Overexpression of FGF1/2 R136E **11**

Purification of FGF1/2 R136E **12**

Fluorescence Spectroscopy **13**

Circular Dichroism Spectroscopy **13**

Time Dependent Trypsin Digestion **14**

Thermal Denaturation **14**

Urea Denaturation **15**

Results and Discussion *15*

Purification of FGF1/2 R136E **15**

Fluorescence and CD Spectroscopy **17**

Time Dependent Trypsin Digestion **19**

Thermal Denaturation **22**

Figure 14. Melting temperature of wild-type and mutant FGF1/2 in the absence and presence of heparin **24**

3.5 Urea Denaturation **24**

Figure 16. Chemical melting temperature of wild-type and mutant FGF1/2 in the absence and presence of heparin. **25**

Conclusions *25*

Literature Cited *27*

Abstract

Fibroblast growth factors are a family of heparin binding proteins that are involved in a broad spectrum of physiological functions, such as angiogenesis, cell proliferation, cell differentiation, and wound healing. Fibroblast growth factor 1 (FGF-1) is a member of the FGF superfamily and can bind universally to fibroblast growth factor receptors. Fibroblast growth factor 2 (FGF-2) is similar in structure to FGF-1 but has been shown to have a potent effect on angiogenesis. A physiologically stable dimerization of these two growth factors could prove to induce desirable effects in the instance of wound healing. In this study, we have designed a FGF1/2 heterodimer composed of FGF-1 and FGF-2 linked together by a glycine linker. We introduced a charge reversal mutation (R136E) within the heparin binding pocket of FGF-1 component of the heterodimer. The variant was expressed using *E. coli* and purified using heparin Sepharose column chromatography. Circular dichroism showed the variant did not alter the backbone confirmation and fluorescence spectroscopy indicated that the tertiary structure of the variant remained intact as well. An increase in resistance to trypsin digestion, thermal denaturation, and urea denaturation was seen in the variant in comparison to the wild-type FGF1/2 protein. This confirms that the introduction of R136E induced overall stability when in comparison to the wild-type. This mutation acts a starting point for the FGF1/2 heterodimer. Future studies will explore the resistance to thrombin and proliferative effects of this mutation as well as examine the mutation in the presence of additional stabilizing mutations.

Keywords: Fibroblast Growth Factors, FGF-1, FGF-2, characterization,

Introduction

Fibroblast Growth Factors (FGFs) are polypeptides that play an essential role in many endocrine signaling pathways as well as various stages of the wound healing process. FGFs are secreted signaling proteins that signal to their respective tyrosine kinases and intracellular non-signaling proteins (Ornitz and Itoh, 2015). FGFs have been established to induce endocrinologic signaling pathways in various phases of the wound healing process by coordinating intracellular signaling pathways while promoting the migration, differentiation, and proliferation of several cell types (Alghanmi, 2017). FGFs, specifically FGF-1 and FGF-2, can influence angiogenesis through both autocrine and paracrine signaling by acting on endothelial cells. FGFs also play a crucial role in the development of the nervous system (Alghanmi, 2017). There have been 22 identified proteins (FGF-1 to 14 and FGF-16 to 23) in humans classified as Fibroblast Growth Factors. These proteins are all structurally similar and have been classified into seven subfamilies based on similarity of structure, function, and gene location: FGF-1 (including orthologs of FGF-1 and 2), FGF-4 (Orthologs of FGF-4, 5, and 6), FGF-7 (Orthologs of FGF-3, 7, 10, and 22), FGF-8 (Orthologs of FGF-8, 17, ad 18), FGF-9 (Orthologs of FGF-9, 16, and 20), FGF-11 (Orthologs of FGF-11, 12, 13, and 14), and the FGF15/ 19 (Orthologs of FGF-15/ 19, 21, and 23) subfamily (Itoh & Ornitz, 2011). FGFs pair with their respective fibroblast growth factor receptors (FGFRs) to mediate processes such as re-epithelialization, matrix deposition, and tissue formation (Alghanmi, 2017). *In vivo*, the major polysaccharide ligand for FGFs are heparan sulfate and heparin (a highly sulfated form of heparan), which occurs as heparan sulfate proteoglycans at the cell surfaces and in the extracellular matrix throughout the body (Kreuger et al, 1999). Interactions between heparan sulfate and FGFs have been shown to be essential for the biological activity of these growth factors (Rapraeger et al).

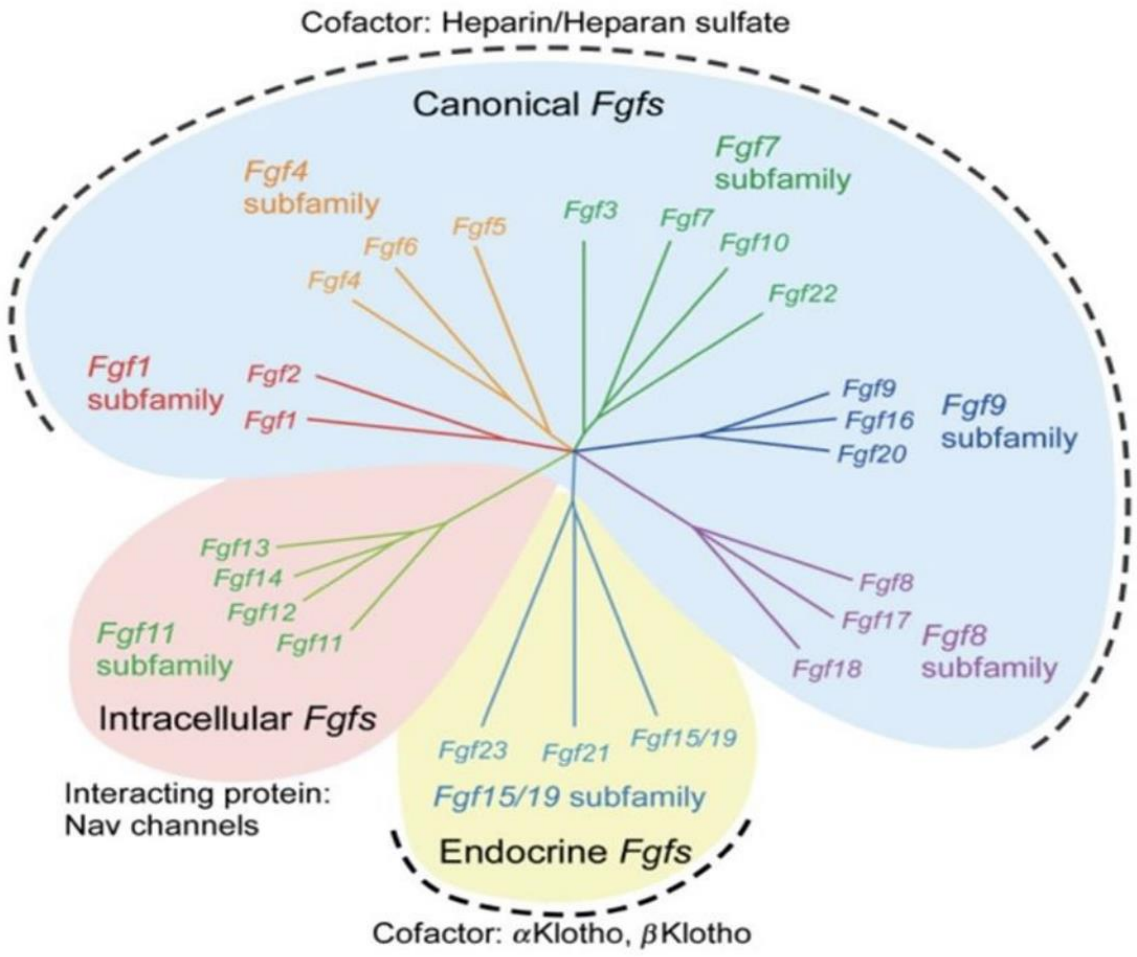

Figure 1. FGF and FGFR families. Depicts the 7 subfamilies containing two to four proteins. Branch lengths are proportional to evolutionary distance between each protein encoding gene. Adapted from, "The Fibroblast Growth Factor signaling pathway," by D. M. Ornitz, 2015, Developmental Biology, 4(3), 215–266. http://doi.org/10.1002/wdev.176 Copyright 2015 by the Authors. WIREs Developmental Biology published by Wiley Periodicals, Inc.

Acidic FGF-1 is a 155 amino acid protein that is a member of the FGF superfamily as it can bind non-selectively to all the four FGF-receptors. FGF-1 is a heparin-binding protein and displays powerful mitogenic properties, due to its binding ability, for cell types such as

hepatocytes, myocytes, endotheliocytes, and neural cells (Alghanmi, 2017). FGF-1 acts on a broad spectrum of biological processes including, angiogenesis, cell differentiation, cell proliferation, and wound healing and is found on the top of the cell surface or within the cell matrix and is released by cells in the event of cellular damage (Alghanmi, 2017). Crystallography of the secondary structure of FGF-1 depicts β trefoil fold structure of 12 beta sheets arranged in an antiparallel direction which are well conserved (Alghanmi, 2017). A heparin binding site is located within the FGF-1. Previous studies have found that protein regulation in signaling transmission and chemical recruitment rely on heparan sulfate proteoglycans (HSPGs) and heparan sulfate glycosaminoglycans (Bellosta et al. 2001).

Fibroblast Growth Factor 2 (FGF-2) is part of a large family of structurally similar proteins that affect growth, differentiation, migration, and survival of a variety of cell types. FGF-2 is an 18 kDa protein that consists of 12 anti-parallel β-sheets organized into a trigonal pyramidal structure (Nugent and Iozzo, 2000). FGF-2 plays a prominent role in the growth and development of new blood vessels and capillaries (angiogenesis) and has been found to regulate the growth and function of vascular cells such as endothelial and smooth muscle cells (Nugent and Iozzo, 2000). FGF-2 also shows mitogenic characteristics, like FGF-1, in that they share roughly 55% of the same amino acid sequence. Unlike FGF-1, FGF-2 is specific for the receptors that it binds. FGF-2 is known to be stored not only in the extracellular matrix, but also in the endothelial cells and fibroblast (Powers et al. 2000). Stimulation of FGF-2 has been suggested to occur in the event of cell damage, death, and non-lethal membrane disruptions (Nugent and Iozzo, 2000).

An effective linkage of FGF-1 and FGF-2 would allow for the characteristics of FGF-1 to be applied in cases where FGF-2 binds to FGF2R. Since FGF-1 has universal binding

capabilities, it would effectively induce its strong proliferative effects along with an increased stability due to FGF-1 high affinity for heparin while also utilizing the angiogenic characteristics applied by FGF-2 at the site of the wound. A glycine linker has been engineered to link FGF-1 and FGF-2. This variant shows susceptibility to thermal, chemical denaturation, as well as thrombin digestion, so a variant with physiologically stable characteristics could prove to be a protein with clinically significant interest.

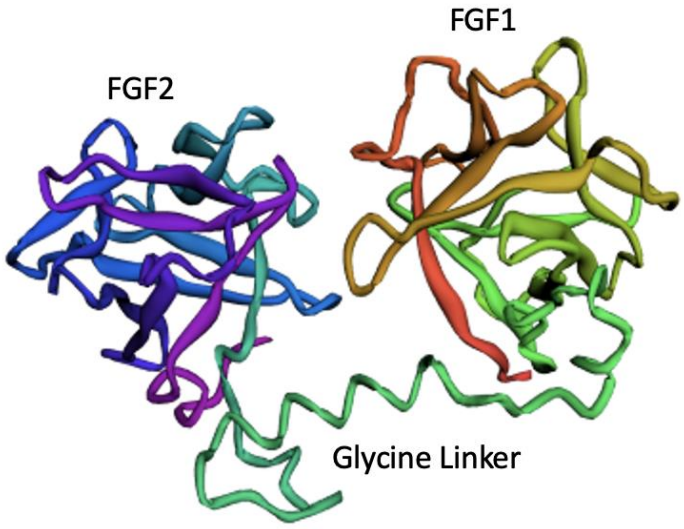

Figure 2. Crystallization of FGF1/2 heterodimer

In this study we investigated the effects of the R136E mutation in FGF-1 of the FGF1/2 heterodimer. Previous studies have found numerous beneficial characteristics of the R136E mutation in hFGF-1 including significant increase in the thermal stability, decrease in heparin binding affinity, increase in the resistance of proteolytic degradation (including complete resistance to cleavage by thrombin by reversing the charge within secondary cleavage site in the heparin binding pocket), and a two-fold increase in cell proliferation activity (Kerr et al. 2019). Mutation R136E in FGF-1 has also been shown to stimulate insulin-independent glucose uptake and could thus show potential as an anti-diabetic drug (Kerr et al. 2019). If similar results in the

FGF1/2 heterodimer with R136E could prove to utilize both the effects of FGF-1 and FGF-2 in the case of cellular damage.

Medicare has found that the treatment for all wound types cost an estimated $96.8 billion including the cost of infection care in 2014 and of those, surgical wounds and diabetic ulcers were found to be the most expensive to treat (Sen, 2019). One major reason that wounding accounts for the highest cost of medical procedures is due to the time that wounds can take to heal. While minor wounds often heal quickly and without complications, wounds such as diabetic ulcers and other chronic wounds can require constant care for the remainder of one's life. With the production of a stable FGF1/2 heterodimer, a therapeutic wound healing agent could be devised to initiate expedited angiogenesis and cellular regeneration which could be used in numerous clinical areas such as chronic wounds, diabetics, and surgical recovery. FGF1/2 heterodimer with R136E could prove to be a stable, physiologically active, protein implemented in a therapeutic topical agent.

Heparin is a negatively charged polysaccharide that is naturally formed and released into blood circulation by the secretory granules of the white blood cells in the instance of tissue damage (Alghanmi, 2017). Heparin displays significant anticoagulant activity and is often used in clinical settings as an intravenous anti-clotting agent. Heparin consists of a linear polysaccharide chain of repeated heterogeneous (sulfated and under-sulfated) disaccharide units with an average molecular weight of 3 to 30 kilo-Daltons (Alghanmi, 2017). The high binding affinity of FGF to heparin has shown to increase the resistivity of proteolytic cleavage, increase the thermal stability, and increase the half-life of FGFs while enhancing the efficacy of the protein by two to three-fold. FGF-1 and FGF-2 fail to bind and activate cell surface FGF

receptors in the absence of heparin, implicating heparin as a necessary part of the regulation of FGF activity (Alghanmi, 2017).

Fibroblast growth factors are involved in many biological signaling pathways by binding to their respective fibroblast growth factor receptor (FGFR). FGFR's are tyrosine kinase receptors that, when activated, result in a cascade of intracellular signaling pathways. The FGFR's consists of an N-terminal extracellular binding domain, a transmembrane domain, and a cytoplasmic domain (Abbass, Asa & Ezzat, 1997). Many FGFs are stored/bound within the extracellular matrix and protected by heparin sulfate proteoglycans from degradation (Newman et al. 2004). FGFs heparin binding pocket shows high affinity for heparin which facilitates the ligand binding of FGF/FGFR. When FGF's bind to their receptor, dimerization of the tyrosine kinase receptors on the plasma membrane occurs, activating the signaling pathway of the receptor by autophosphorylation of the receptor within the cytoplasmic domain. Phosphorylated tyrosine receptors then activated one of three main pathways, RAS/MAPK, PI3k/AKT, and PLCy (Majid, et al. 2020). The activation of FGFRs induces downstream cascade of effects including cell proliferation, differentiation, and angiogenesis.

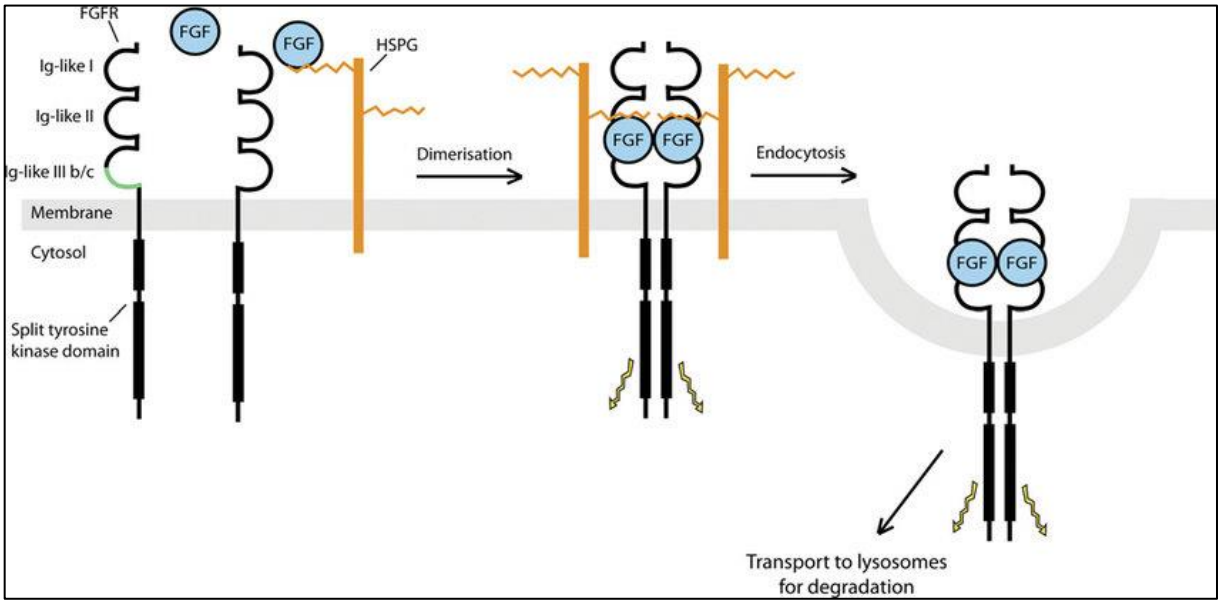

Figure 3. Depiction of FGF binding mechanism. FGF is brought to the FGFR while bound to heparin. The FGF ligand binds to the FGFR and pulls the two tyrosine kinases together. This induces phosphorylation of the cytoplasmic domain of the FGFR, which recruits proteins involved in cellular proliferation. Adapted from, "Fibroblast growth factors and their receptors in cancer" by J. Wesche, K. Haglund & E. Haugsten. (2011). *The Biochemical journal*. 437. 199-213. 10.1042/BJ20101603.

Limitations in previous designs of FGF-1 as a therapeutic agent center around FGF-1's susceptibility to proteolytic cleavage. Although FGF-1 lacks a specific thrombin cleavage site (LVPRGS), a secondary site is located within the protein that is susceptible to cleavage (Erzurum et al. 2003). FGFs are also sensitive to thermal denaturation. Previous studies have found that the introduction of a negative charge at the 136 location within FGF-1 (R136E) has been able to provide complete resistance to thrombin secondary cleavage as well as significantly increase the thermal resistance of FGF-1 (Kerr et al. 2019). In this study, assessment of the heterodimer's stability gives insight into the potential the mutant shows for being implemented into a therapeutic agent to assist in the healing of wounds.

Methods and Materials

Overexpression of FGF1/2 R136E

The overexpression of FGF1/2 R136E was essential in producing a large amount of protein that was then used to conduct subsequent experimental characterizations. To begin this procedure, we added two 1mL glycerol stock and 200 uL of kanamycin to 200mL of sterile

autoclaved LB media. The solution was then allowed to incubate overnight (13-14 hours). Once the initial incubation was complete, 25 to 30 mL of this solution and 500 uL of kanamycin were then added to four 500 mL flasks containing sterile autoclaved LB media. This solution was then allowed to incubate for 3 hours to allow for the bacteria to enter the log phase. All flasks were then inundated with 1mM (500 uL) Isopropyl beta-D-1-thiogalactopyranoside (IPTG). This induced the *Lac operon*, triggering faster protein expression. The cells were then allowed to incubate for 3.5 to 4 hours. After this incubation, the cells were collected into centrifuge tubes and harvested by centrifugation at 6000 rpm for 20 minutes at 4 degrees Celsius. After this centrifugation, the supernatant was discarded, and the cells were resuspended in 35mL of 10mM Phosphate buffer into 50 mL falcon tubes. Once the cells were resuspended in the falcon tubes, the cells were centrifuged at 6000 rpm for 25 minutes at 4 degrees Celsius. After centrifugation, the supernatant was discarded, and the cells were stored at -20 degrees Celsius until further purification.

Purification of FGF1/2 R136E

To purify the sample protein, affinity chromatography separation was used. This procedure utilizes a heparin-Sepharose column and was used to purify FGF1/2 R136E. First, the pellet from the initial overexpression was resuspended in 10mM Phosphate buffer. Once resuspended, the cells were ruptured using sonication with parameters of 10 seconds of sonication followed by 10 seconds rest for 22 minutes. Before beginning the sonication, the column was equilibrated with 10mM Phosphate buffer. Once the sonication was completed, the cells were collected in small centrifuge tubes and centrifuged at 19,000 rpm for 30 minutes at 4 degrees Celsius. Once the centrifugation was completed, the supernatant was collected into a

falcon tube and loaded into the column. Once the supernatant was loaded into the column, the column was then washed with an increasing incrementation of salt concentrations (100mM, 300mM, 500mM, 800mM, 1250mM, 1500mM NaCl). The fractions were collected and then run through an SDS-PAGE gel to determine the concentrations at which the protein eluted. The fractions at which the protein eluted (1500mM) were then buffer exchanged using G8 Phosphate buffer, and a 10kDa MW centrifugal concentrator and centrifuged at 4500 rpm at 4 degrees Celsius. After determining the concentration of FGF1/2 R136E protein using NanoDrop at A280 nm, the protein was collected into 1 mL Eppendorf tubes and stored at -20 degrees Celsius for further characterization.

Fluorescence Spectroscopy

Using the JASCO 1500 spectrometer, the tertiary structure of FGF1/2 R136E was observed in comparison to the wild-type FGF1/2. A 1 mL sample of protein was loaded into a crystalline cuvette and inserted into the spectrometer. Absorbance analysis was conducted over a range of 300 to 450 nm.

Circular Dichroism Spectroscopy

Using the JASCO 1500 CD spectrometer, the secondary structure of the variant protein was assessed using circular dichroism spectroscopy and then compared to wild-type protein circular dichroism spectra. A 1 mL sample protein was loaded into a crystalline cuvette and inserted into the spectrometer; Absorbance was measured over 250 to 190 nm.

Time Dependent Trypsin Digestion

To observe the backbone flexibility of FGF1/2 R136E, a time dependent trypsin digestion was performed and compared to the wild-type FGF1/2. The digestion was performed for both proteins, wild-type and mutant FGF1/2 in the presence and absence of heparin.

A 2.5 mg/ml concentration of trypsin was used to conduct the digestion. Ten samples were prepared in Eppendorf tubes with 15 uL of protein and 75 ul of 10 mM Phosphate buffer. Time exposures of 2, 4, 6, 8, 10, 30, 45, and 60 minutes were used to assess the extent of digestion. To begin the digestion, 10 uL of trypsin was added to the tested tube and immediately added to a 37 degrees Celsius water bath. Once the time parameter was met, the tube was removed from the bath and 10 uL of 10% trichloroacetic acid (TCA) was added to stop the digestion. Once all the samples were collected, an SDS-PAGE was run to determine the extent of the digestion. Using the image of the gel from the SDS-PAGE, a densitometric scanning analysis was used to quantify the results of the digestion.

Thermal Denaturation

To evaluate the thermal and conformational stability of FGF1/2 R136E, Differential Scanning Calorimetry (DSC) was used to determine the melting point (Tm) of the protein. The Tm was determined for FGF1/2 wild-type and R136E in the absence and presence of heparin. To conduct this assay, the DSC was first conditioned with G8 Phosphate buffer. Once conditioned, the protein of interest was loaded in the well, pressurized to 44 ATM and then allowed to heat from 20-80 degrees Celsius. Curved plots, graphing the temperature on the x-axis and the energy released on the y-axis, were recorded with the peak indicating the Tm of the protein (50% of the sample is in the denatured state).

Urea Denaturation

Using the JASCO 1500 CD spectrometer, a 1 mL sample of protein was loaded in a 1cm path cuvette. The folding and unfolding nature of the wild-type and R136E FGF1/2 were recorded while denaturation was induced by addition of increasing concentrations of 8M urea. Absorbance values were obtained over a range of 300 to 450 nm. Once the values were obtained, the percent unfolded protein was plotted against the denaturant concentration.

Results and Discussion

Purification of FGF1/2 R136E

To obtain protein samples, purification using affinity chromatography was used. Pure samples were eluted from the heparin-Sepharose column by increasing the NaCl concentrations. For FGF1/2 wild-type and R136E, concentration of 100 mM NaCl, 300 mM NaCl, 500 mM NaCl, 800 mM NaCl, 1250 mM NaCl, and 1500 mM NaCl were used to elute protein. FGF1/2 wild-type and R136E were found to both elute at high salt concentrations (800, 1250, and 1500 mM NaCl), which indicate that the heparin binding pocket within FGF1/2 R136E is still largely intact, however, elution of FGF1/2 R136E was seen at 800 mM NaCl and 1250 mM NaCl more significantly than in wild-type FGF1/2, suggesting that the presence of degraded monomers was slightly higher. At high salt concentrations, the electrostatic interactions between the heparin-Sepharose and FGF1/2 are broken down and the protein is eluted. An SDS-PAGE analysis was conducted to observe if the protein was eluted at the correct concentrations, as well as to ensure that samples used in the buffer exchange contain pure protein.

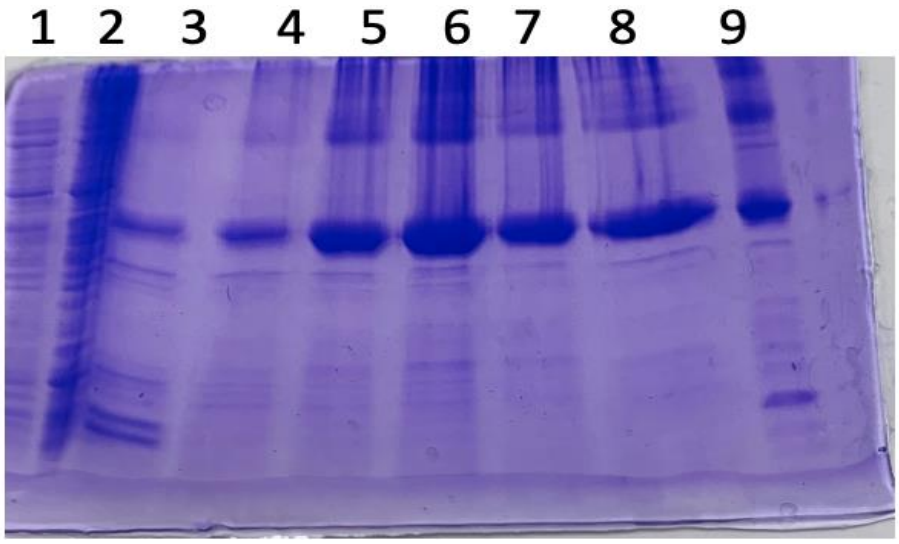

Figure 4. The SDS-PAGE analysis of the FGF1/2 R136E mutant using a heparin-Sepharose column. The black box indicates elution of protein and lane 9 indicates the target protein weight. Lane 1: supernatant. Lane 2: unbound. Lane 3: 1250 mM NaCl. Lane 4-8: 1500 mM NaCl. Lane 9: protein marker.

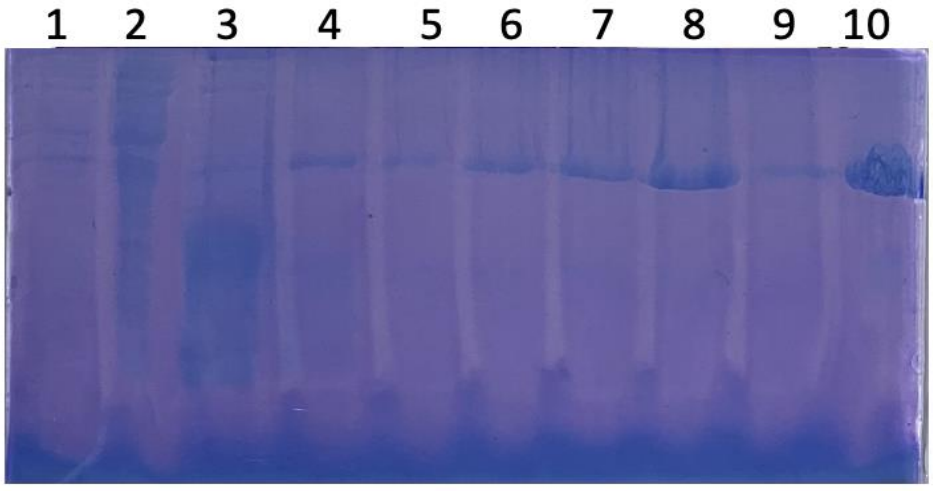

Figure 5. The SDS-PAGE analysis of the FGF1/2 wild-type using a heparin-Sepharose column. The black box indicates the band which corresponds to the desired protein. Lane 1: supernatant. Lane 2: unbound. Lane 3: 800 mM NaCl. Lane 4-9: 1500 mM NaCl. Lane 10: protein marker.

Fluorescence and CD Spectroscopy

Fluorescence spectroscopy was conducted on the protein collected from the previous purification. FGF1/2 have exposed tyrosine residues while in their native conformation. Taking advantage of tyrosine residual fluorescence capabilities, fluorescence spectroscopy could be used to determine if the target protein was within its native conformation. Tyrosine's fluoresce from a range of approximately 305 to 310 nm, while tryptophan, an amino acid within the interior of the native conformation of FGF1/2, fluoresces at approximately 360 nm. By determining the fluorescent peaks of both the wild-type and R136E FGF1/2, it can be determined whether the protein of interest is folded within its native conformation. Using the JASCO 1500, sample absorbance of both the wild-type and R136E FGF1/2 were collected and then plotted against each other. The results show a peak at the desired range for tyrosine (Figure 6.), indicating that FGF1/2 R136E has maintained its proper tertiary structure.

Circular dichroism was conducted on the sample. FGF1 and FGF2 are similar in their secondary structure. FGF proteins contain twelve beta strands organized in the central domain, five of which form a hairpin binding structure, that are organized in an antiparallel direction and are well conserved (Brych, Blaber, Logan & Blader). The curve of the CD spectra data (Figure 7.) that was collected indicates that there is a large presence of beta sheets detected within the wild-type protein, and when overlayed with the R136E variant, the general structure appears to be intact.

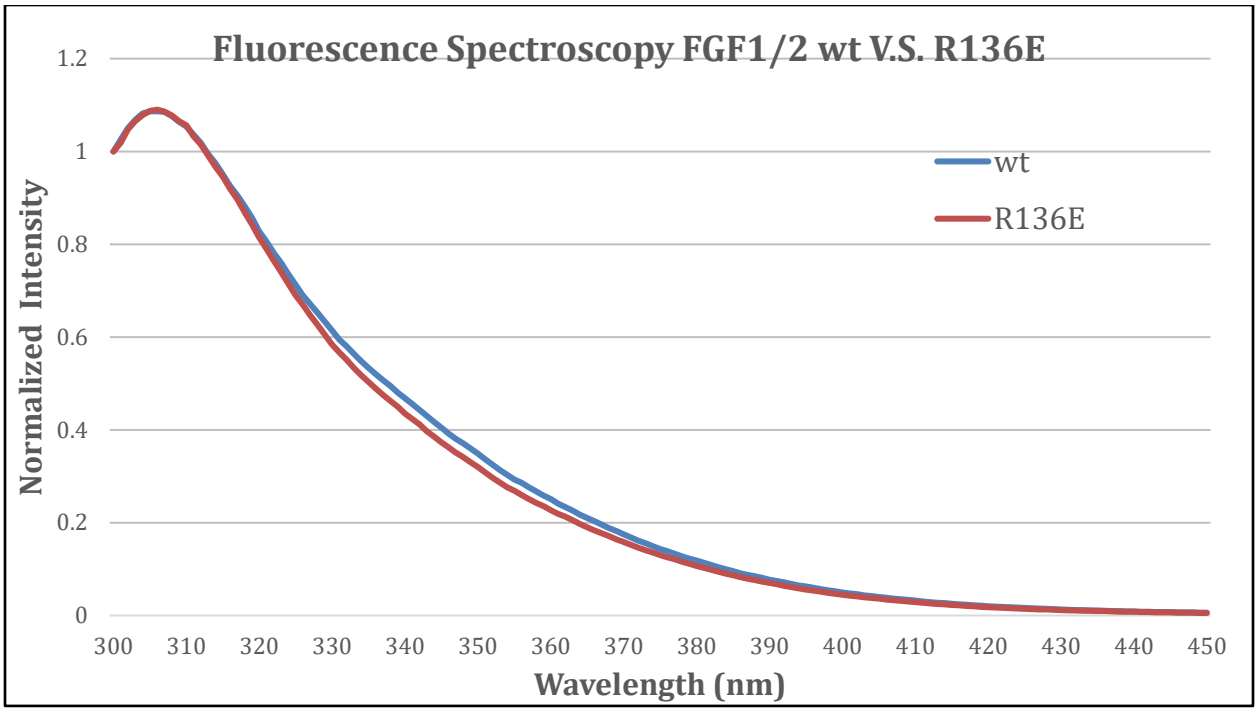

Figure 6. Fluorescent absorbance over the range of 300 to 450 nm of FGF 1/2 wild-type and R136E.

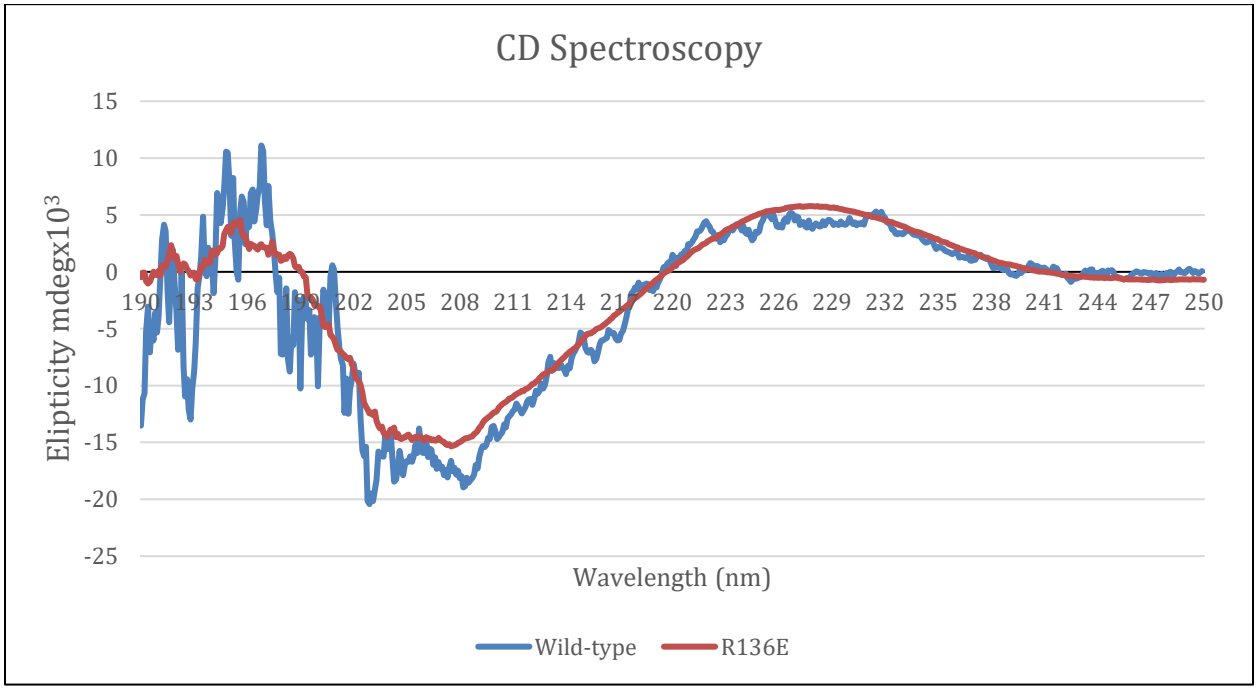

Figure 7. Circular Dichroism absorbance over the range of 250 to 190 nm of FGF1/2 wild-type and R136E.

Time Dependent Trypsin Digestion

A trypsin digestion experiment was conducted to examine the changes in the stability and backbone flexibility of our protein against proteolytic cleavage. To observe the effects of proteolytic cleavage by trypsin, a time dependent examination was used. Trypsin cleaves lysine and arginine at the carboxyl terminal of the amino acids. The R136E mutation also induces a negative charge within the positively charged binding site of FGF-1, decreasing the flexibility of the heparin binding pocket and making potential trypsin cleavage sites less accessible to trypsin digestion (Kerr et al. 2019). The mutation R136E also removes one of the cleavage sites of trypsin, so it is expected to have a greater proteolytic resistance than that of the wild-type. Both the wild-type and R136E FGF1/2 were subjected to time dependent trypsin digestion in the presence and absence of heparin. An SDS-PAGE analysis was then conducted and analyzed using densitometric scanning.

The data shows that the mutation R136E confers greater resistance to the proteolytic cleavage of trypsin compared to the wild-type in the absence of heparin. Digestion of R136E by trypsin in the absence of heparin shows nearly a 50% reduction in degradation compared to that of the wild-type at 2 minutes. At 4, 6, and 8 minutes, the mutant is nearly 6 times more resistant to proteolytic cleavage than the wild-type. After 10 minutes, there is a drop-off in resistivity and by 45 minutes nearly 100% of both the wild-type and FGF1/2 R136E are digested.

In the presence of heparin, both wild-type and R136E showed a significant increase in proteolytic cleavage resistance. The densitometric scans show that 40% of the protein was digested after 45 minutes. Compared to the same parameter without heparin, 83% of the protein

was digested. This indicates that in the presence of heparin, FGF1/2, and FGF1/2 R136E both bind to heparin and lose backbone flexibility.

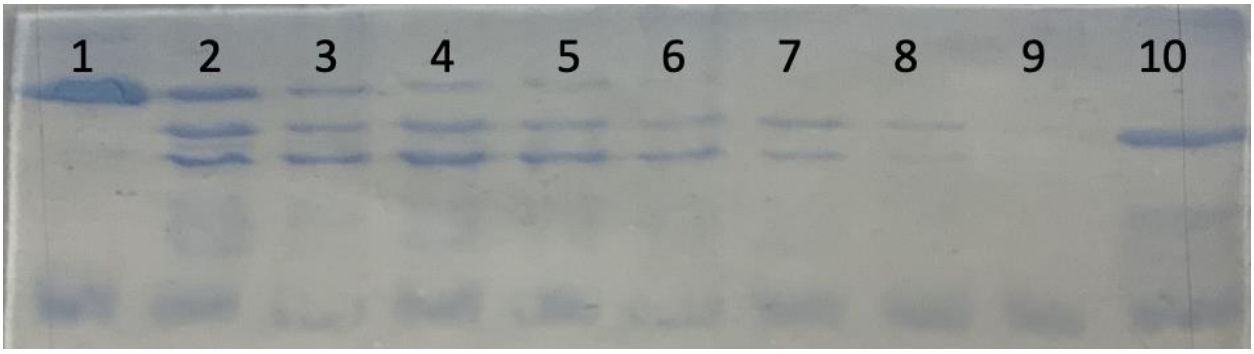

Figure 8. SDS-PAGE of wild-type FGF1/2 after trypsin digestion. Lane 1: protein control. Lane 2: 2 mins. Lane 3: 4 mins. Lane 4: 6 mins. Lane 5: 8 mins. Lane 6: 10 mins. Lane 7: 30 mins. Lane 8: 45 mins. Lane 9: 60 mins. Lane 10: trypsin control.

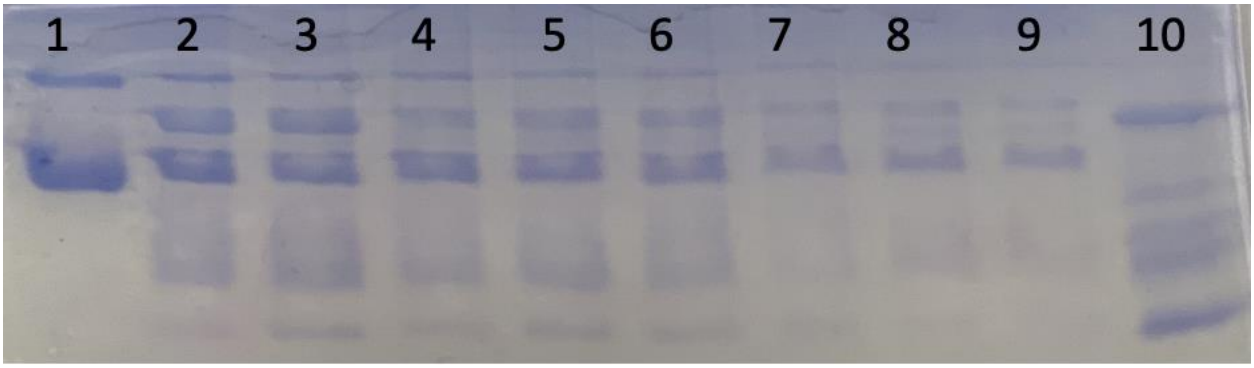

Figure 9. SDS-PAGE of FGF1/2 R136E after trypsin digestion. Lane 1: protein control. Lane 2: 2 mins. Lane 3: 4 mins. Lane 4: 6 mins. Lane 5: 8 mins. Lane 6: 10 mins. Lane 7: 30 mins. Lane 8: 45 mins. Lane 9: 60 mins. Lane 10: trypsin control.

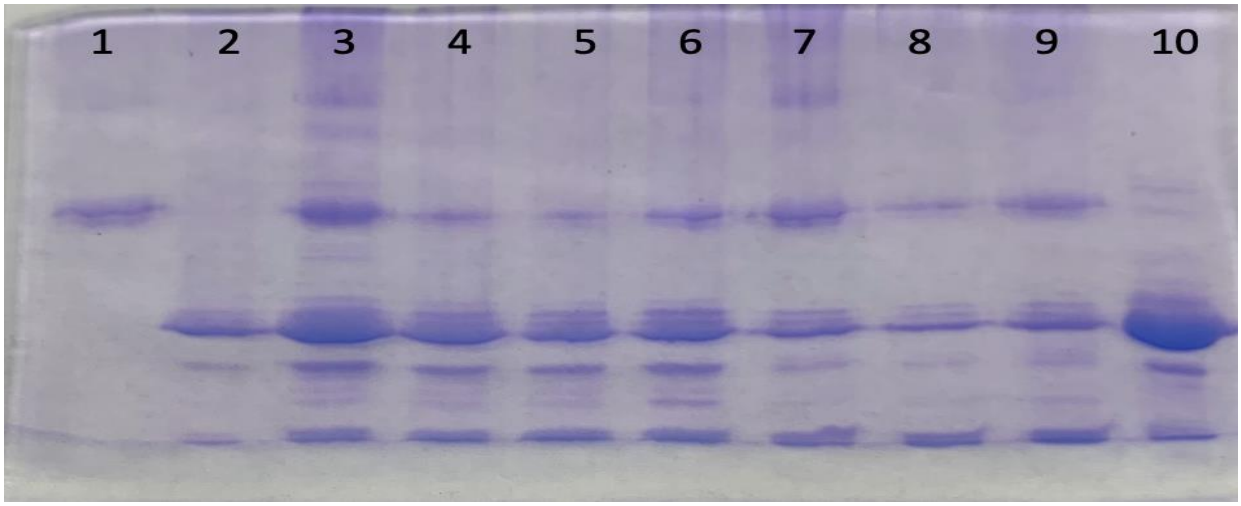

Figure 10. SDS-PAGE of wild-type FGF1/2 after trypsin digestion **in the presence of heparin**. Lane 1: protein control. Lane 2: 2 mins. Lane 3: 4 mins. Lane 4: 6 mins. Lane 5: 8 mins. Lane 6: 10 mins. Lane 7: 30 mins. Lane 8: 45 mins. Lane 9: 60 mins. Lane 10: trypsin control.

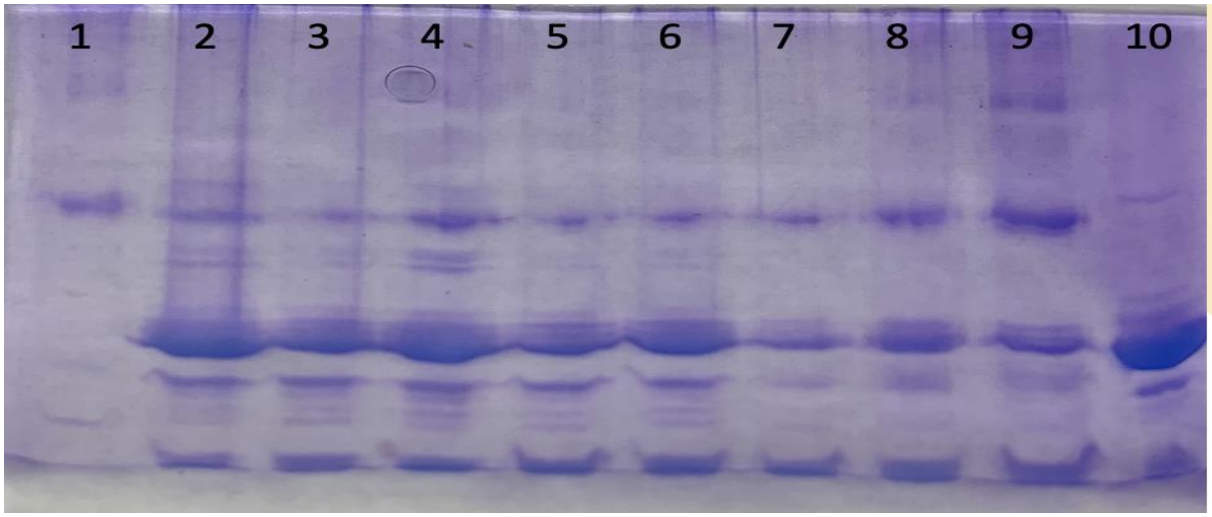

Figure 11. SDS-PAGE of R136E FGF1/2 after trypsin digestion **in the presence of heparin**. Lane 1: protein control. Lane 2: 2 mins. Lane 3: 4 mins. Lane 4: 6 mins. Lane 5: 8 mins. Lane 6: 10 mins. Lane 7: 30 mins. Lane 8: 45 mins. Lane 9: 60 mins. Lane 10: trypsin control.

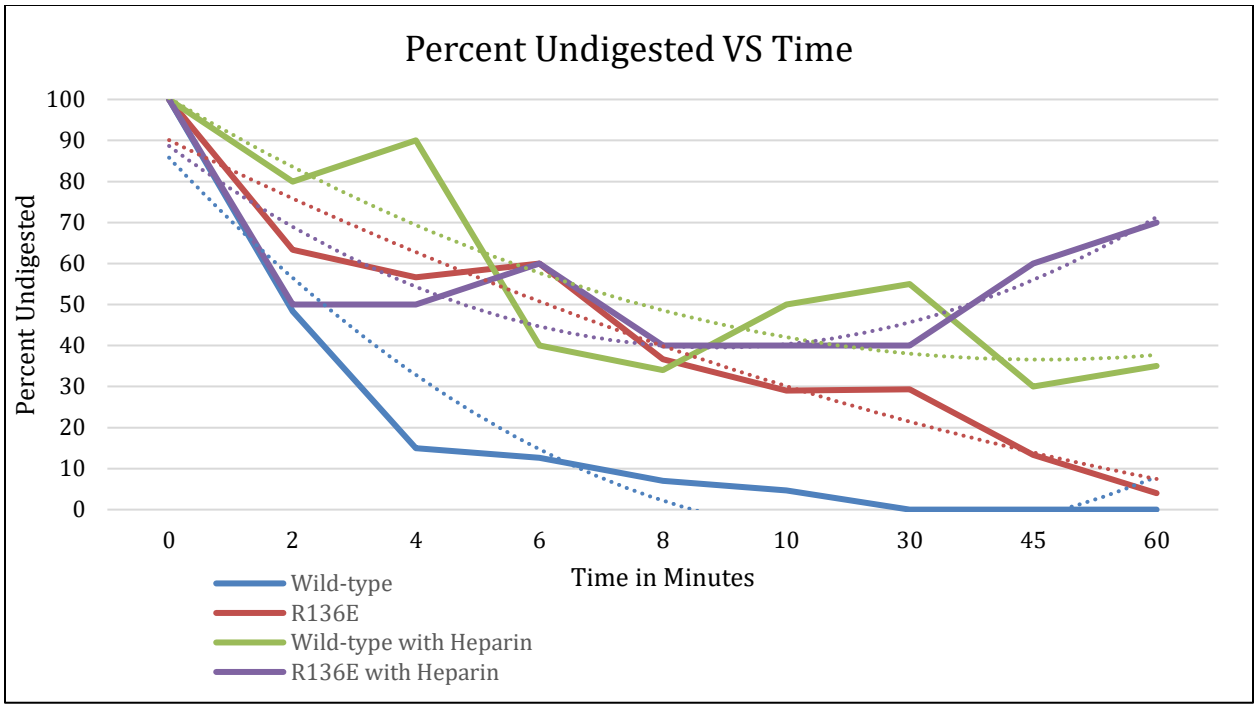

Figure 12. Densitometric scans of the wild-type and R136E FGF1/2. The percent protein is plotted on the Y-axis with the time parameter plotted on the X-axis.

Thermal Denaturation

Differential Scanning Calorimetry was used to determine the effects of the R136E mutation in FGF1/2 on the thermal stability of the protein. The R136E mutation is located within the heparin binding pocket of the FGF1/2. Heparin stabilizes the native protein by providing greater thermal denaturation resistance.

The DSC was performed on the wild-type and R136E mutant in the presence and absence of heparin. In the absence of heparin, the wild-type showed a Tm of 45.8 degrees Celsius, while the R136E mutant showed a 7.8-degree Celsius increase to a Tm of 53.6 degrees Celsius. In the presence of heparin, the wild-type showed a Tm of 53.7 degrees Celsius while the R136E mutant showed an increase of 16.1 degrees Celsius to 69.8 degrees Celsius. These results show that

thermal stability in the absence of heparin is improved with the R136E mutation and even more improved in the presence of heparin.

The mutation R136E is centrally located within the heparin binding pocket of FGF1 of the FGF1/2 heterodimer. A plausible explanation to why thermal resistance is increased could be explained by the introduction of a negative charge in the positive binding region creating a counter-ion effect and allows for the repulsion within the heparin binding pocket of FGF1 subunit to be partially nullified (Kerr et al. 2019).

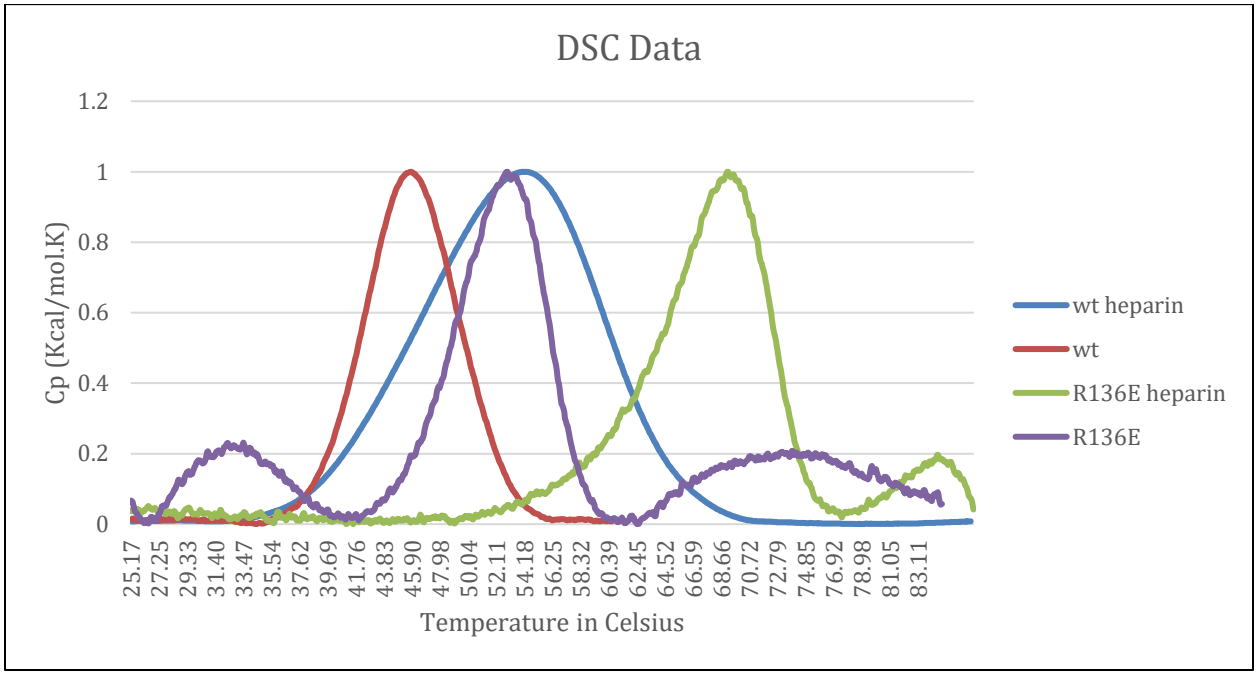

Figure 13. The thermal denaturation profiles of FGF1/2 wild-type and R136E in the absence and presence of heparin.

	wt FGF1/2	R136E FGF1/2
T_m without Heparin	45.8	53.6
T_m with Heparin	53.7	69.8

Figure 14. Melting temperature of wild-type and mutant FGF1/2 in the absence and presence of heparin.

3.5 Urea Denaturation

A urea denaturation was conducted to observe the susceptibility of FGF1/2 R136E to denaturation compared to wild-type FGF1/2. The results indicate that the R136E mutation increases the proteins resistance to chemical denaturation by urea. In the presence of heparin, the variant showed a substantial increase in resistivity to chemical denaturation by urea. The introduction of R136E increased the Cm from 2.64 to 2.9 in the absence of heparin. In the presence of heparin, the Cm increased from 3.45 to 4.59 with the introduction of R136E.

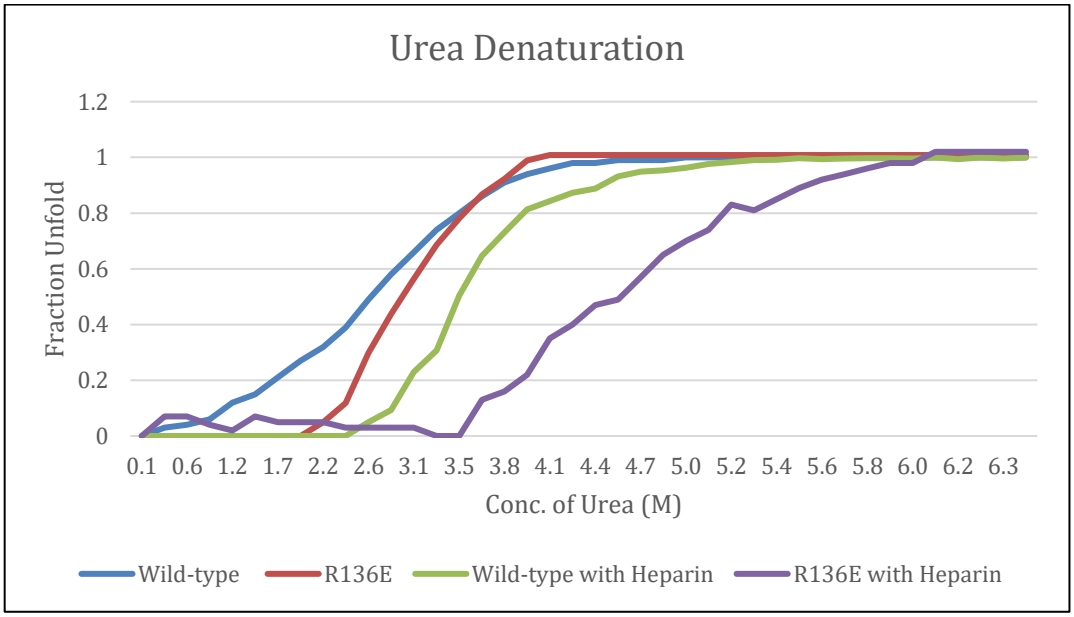

Figure 15. Urea denaturation data plotted with percent denatured vs the concentration of urea. Data represents the wild-type, R136E mutant of FGF1/2 in the absence and presence of heparin.

Cm wt	Cm R136E	Cm wt w/ Heparin	Cm R136E w/ Heparin
2.64	2.9	3.45	4.59

Figure 16. Chemical melting temperature of wild-type and mutant FGF1/2 in the absence and presence of heparin.

Conclusions

Based on the results, the introduction of a negative charge within the heparin binding pocket of FGF1 within FGF1/2 by R136E variant has shown to introduced overall stability within the heterodimer. The R136E variant has shown to maintain proper secondary and tertiary structure, while increasing its proteolytic, thermal, and chemical resistance. These results indicate that FGF1/2 R136E shows promise as a stable heterodimer and with further assessment could be implemented into a therapeutic treatment to aid in the acceleration of wound healing in a clinical setting.

In future studies, it would prove necessary to evaluate the thrombin resistance of FGF1/2 R136E. Theoretically, the secondary cleavage site within FGF-1 of the FGF1/2 heterodimer is no longer present, so FGF1/2 R136E should show complete resistivity to thrombin cleavage. This is essential to examine, due to the presence of thrombin at wound sites where the FGF1/2 heterodimer would be implemented. It would also be beneficial to evaluate the cell proliferative

effects of FGF1/2 R136E to assess whether cell proliferative effects, that have been observed in the previous FGF-1 R136E study, have been maintained. If the proliferative effects and thrombin resistance have been maintained, FGF1/2 R136E will show great promise as a key mutation to increase the thermal, chemical, and proteolytic resistance of FGF1/2. This mutation could be accompanied with additional mutations to provide the greatest overall stability of the FGF1/2 heterodimer and be implemented into therapeutics to aid in the healing of chronic, surgical, or acute wounds.

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