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Abstract

Anginex is a cytokine-like β-sheet forming peptide of 33 amino acids with potent anti-angiogenic activity. Anginex is essential in inhibiting abnormal processes caused by angiogenesis, such as tumor growth and blood vessel formation. However, Anginex has limitations, including poor stability, short half-life, complicated synthesis, and low purity. Rubredoxin dimer (RdRd) is used as a protein tag to improve stability and detection of Anginex during purification for the first time. A plasmid was designed to contain the RdRd-Anginex fusion protein. RdRd-Anginex plasmid was transformed and expressed in *E. coli* BL21star cells. The results show RdRd-Anginex has been purified using a Nickel Sepharose column. Enterokinase, a proteolytic enzyme, was then used to cleave between the RdRd from Anginex. The cleaved sample was placed on a Nickel Sepharose column where the Anginex sample was eluted.

Profiles of the Authors

Suraj Kolluru graduated from the University of Arkansas in 2022, where he obtained a degree in Biochemistry from the Fulbright College of Arts & Sciences.

Dr.Patience Salvalina Okoto. Research Associate at Arkansas Integrative Metabolic Research Center. She is interested in metabolic studies, protein structure and dynamic and protein purification.

Dr. Thallapuranam is a professor of Chemistry & Biochemistry with significant interest in design of novel fibroblast growth factor (FGF)-based rational design of therapeutic principles for chronic wound care, anti-diabetes, anti-obesity, and osteoporosis. His research is supported by several Federal grants (NIH/DOE/NSF/USDA). He currently serves as the NIH sponsored AIMRC Bioenergetics core. He also currently serves as the Director of the Office of Undergraduate Research (in DRI). He has published about 10 US patents and 175 peer-reviewed research papers in highimpact Journals. He is the recipient of several teaching, research, and service awards including the Honors College Distinguished Faculty award, Honors College Faculty Gold medal, Collis Geren Outstanding Interdisciplinary Graduate Faculty award, elected as the member of the National Academy of Inventors, ARSC Master Teacher award. Golden Tusk Award, and the prestigious Nadine Baum Award for outstanding teaching. He is also the member of the University of Arkansas Teaching Academy. Dr. Thallapuranam also regularly serves on the grant proposal review panel of Federal agencies such as NIH, NSF, and DOE. He currently serves as the Associate Editor of the Frontiers of Molecular Biophysics journal and on the Editorial Board of several peer-reviewed journals including the prestigious Journal of Biological Chemistry.

Dr.Zeina Alraawi is a postdoc at Chemistry and Biochemistry department/Arkansas university. She is interested in analysis the cell signaling of cytokines, protein therapeutics, utilizing biophysical techniques in structural protein analysis.

Introduction

Angiogenesis is the formation of blood vessels from the existing vasculature. It involves the migration, growth, and differentiation of endothelial cell. It is essential for delivering oxygen to various tissues throughout the body. Although it is pivotal to human function, it can potentially lead to abnormal processes like tumor metastasis, arthritis, retinoids, and diabetic retinopathy [1]. Anginex is a cytokine-like β-sheet forming peptide 33 amino acids peptide with potent anti-angiogenic activity [2]. The search for angiogenic inhibitors has centered on controlling two processes that promote angiogenesis: 1) endothelial cell (EC) growth and 2) adhesion. EC is more accessible to pharmacological agents delivered via the blood than other cells. EC is also genetically stable and is not easily mutated into drug-resistant variants [1].

Anginex has been designed by utilizing basic folding principles and incorporating short sequences from β-sheet domains of anti-angiogenic agents: platelet factor-4, interleukin-8, and bactericidal-permeability increasing protein (BPI) [3]. Recent studies have indicated that Anginex might be utilized in gene therapy and radiation procedures to inhibit tumor growth [2,4]. Anginex has an amphiphilic and tetramer conformation. Anginex binds to the endothelial cell (EC) membrane and disrupts lipid bilayers through micellization and pore formation [5,6].

Anginex has been shown to help enhance the inhibitory effects of chemotherapy, and radiotherapy, and it has been shown that Anginex, with a suboptimal dose of radiation, can cause tumors to regress to an impalpable state [7]. Recombinant proteins were first synthesized in 1982 when recombinant human insulin became the first medication through recombinant DNA technology.

Recombinant proteins are expressed in a heterogeneous host to synthesize various drugs and conduct research [8]. However, there are many limitations which include low expression yields, difficult localization of the desired protein, and inclusion body formation. Inclusion bodies are misfolded proteins that are functionally inactive and insoluble. Many isolated proteins must be maintained at a specific pH and temperature to keep structure and functionality. If not placed in the appropriate environment, protein

aggregation may result [2,8,9].

Rubredoxin is a class of iron-containing metalloprotein with 80 acids, has 7KDa molecular weight, and is highly thermostable. It is characterized by having 4 conserved cysteine residues that coordinate an iron atom through the sulfur atom of the oxxCzthiol side chain [10]. Rubredoxin improves the efficiency of the expression and purification process by providing stability and easy visualization of the target protein due to its red color. The limitations of Anginex purification include poor stability, short half-life, complicated synthesis, and low purity. In this context, Rubredoxin dimer was used as a protein tag to enhance the purification of Anginex [10,11].

In our research, we used (RdRd) as a protein tag to purify Anginex. We aimed to utilize RdRd as a tag for Anginex to improve its stability, increase its half-life, and purify it with fewer contaminations. Moreover, Anginex might be used in pharmaceutical manufacturing.

Methods

A pET 22b vector encoding for expression of recombinant RdRd-Anginex fusion protein was transformed into BL21* (DE3) *E. coli* cell. Transformation was performed by adding 3 ml of plasmid to 100 ml of BL21* (DE3)component cells in an Eppendorf tube. The solution was placed on ice for 30 minutes and transferred to 42°C for 45 seconds to heat shock the bacteria cells, allowing entry of the plasmid into the host cell. The solution was placed back on ice for 3 minutes. 800 mL Lysogeny broth (LB) media was added to the mixture for incubation on a New Brunswick Science Innova 4330 Refrigerated Incubator shaker at 37°C for 45 min at 225-250 rpm. Using a sterile spreader, 100 mL of the solution was spread on three LB agar plates with ampicillin. Once spread, the LB agar plates were placed upside down in an incubator for 12-16 hours.

Small scale expression: After incubation, a single colony of bacteria was transferred to 10 mL of sterile LB media with 10 mL of 100 mg/mL ampicillin where it

grew for 12-16 hours in the New Brunswick Science Innova 4330 Incubator shaker at 37°C. 10 mL of sterile LB media was inoculated with 500 μL overnight culture and 10 mg/mL ampicillin. The unused overnight culture was placed in the -80°C freezer. The overnight culture was placed in the same refrigerated shaker for 1 to 1.5 hours. The optical density (OD) was determined by taking a 1 mL aliquot of the culture and measuring the absorbance at 600 nm using a UV-Vis Spectrophotometer. Once the desired OD of the culture reached (0.6 - 0.8.), 9 μL of 1 M isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the culture and incubated in the same refrigerated incubator for 3.5 hours.. After the culture was incubated, it was centrifuged in a Thermo Scientif Sorvall Lynx 6000 centrifuge at 4500 rpm at 4°C for 15 minutes. The supernatant was separated from the pellet. The cell pellet was resuspended in buffer and sonicated in 10 second intervals using a Branson Sonicator 150 for 3 minutes. The sonicated cells were centrifuged in a Thermo Scientific Sorvall Legend Micro 21 Microcentrifuge at 13,000 rpm for 5 minutes. Samples from pre- and post-induction with IPTG were taken for the supernatant and pellet. The samples were prepared on a 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis to verify the presence of RdRd-Anginex. The SDS-PAGE gel should demonstrate a molecular weight around 17 kDa to verify the presence of RdRd-Anginex. Glycerol stocks were prepared by adding 3.5 mL of autoclaved 60% glycerol solution containing a bacteria culture to a falcon tube. 1 mL aliquots of the solution were placed in Eppendorf tubes and stored in an -80°C freezer.

Expression was performed with one 1L Erlenmeyer flask and four 2L Erlenmeyer flasks. 5g of LB media and 200 mL of DI water were added to the 1L flask, while 12.5g of LB media and 500 mL of DI water was added to each 2L flask. The flasks were autoclaved in a Thermo Scientific SG-120 Autoclave Sterilizer for 45 minutes at 121°C and 15 psi. After the flasks cooled down, two RdRd-Anginex glycerol stocks and 200mL of 100 mg/mL ampicillin were inoculated in 1L flask. The inoculated culture in the 1L flask was placed in the New Brunswick Scientifics Innova 4330 refrigerated incubator shaker where it was incubated 37°C for 14-16 hours. After incubating, 30 mL of the overnight culture was added into each of the four 2L flasks with 500 mL of Ampicillin. The four flasks were

placed on the incubated shaker for 3.5 to 4 hours until the optical density (OD) reaches 0.6 to 0.8. Isopropyl-b-D-galactosidase, IPTG, was added before incubation to allow for cell growth. The culture was transferred into centrifuge tubes and centrifuged at 6000 rpm for 25 minutes at 4°C in a Thermo Scientific Sorvall Lynx 6000 centrifuge with a JA-10 rotor. The supernatant from each of the centrifuge tubes was discarded. The pellets formed were suspended in 10-12 mL of 10 mM Phosphate buffer. The mixture was transferred to falcon tubes, which were centrifuged once again. The falcon tubes were centrifuged under the same parameters as before. After centrifuging, the supernatant was discarded and stored in the -20°C freezer until needed.

For purification, the pellets were sonicated in 35-40 mL of 10 mM Phosphate Buffer using a Scientific Industries Vortex-Genie 1 Touch Mixer and Branson Sonifier 150. Purification of RdRd-Anginex was accomplished through the Immobilized Metal Affinity Chromatography (IMAC) method using a Nickel-Sepharose column. The column was pre-equilibrated with deionized water and 10 mM Phosphate buffer before loading the sample. The recombinant RdRd-Anginex contains a 6 residue N-terminal histidine tag, which has a high affinity to the Ni^{2+} resin in the column. The supernatant was loaded onto the column at a 0.5 mL/min rate, and a Bio-Rad UV-Vis detector was used to monitor protein at 280 nm. RdRd-Anginex binding to the Ni^{2+} resin was apparent by the red and purple color in the column. Once all the supernatant has been loaded, 10 mM Phosphate Buffer was passed through the column at 1.5 mL/min until the absorbance baselines. Ni^{2+} resin Ni

with increasing concentrations of imadizole (20 mM, 50 mM, 100 mM, 250 mM, and 500 mM) to remove non-specific binding proteins and elute RdRd-Anginex.

Enterokinase Cleavage of RdRd-Anginex**:** Enterokinase is a proteolytic enzyme employed to cleave affinity tagged recombinant proteins. Enterokinase is produced by the cells of the duodenum in the small intestine and is involved in human digestion. It plays a role in activating trypsinogen to trypsin. Through activating the trypsinogen, it indirectly activates pancreatic digestive enzymes. Enterokinase cleaves peptides/proteins on the carboxyl side of lysine after lysine and the site-specific cleavage sequence is Asp-Asp-Asp-Lys. In this experiment, enterokinase was used to cleave the RdRd tag from the Anginex protein through breaking the peptide bond between alanine and aspartate as shown in Figure 1. In the figure, the cleavage site

is indicated by "||." The red colored text represents the RdRd tag while the black colored text represents the Anginex protein (Figure 1).

MHHHHHHMAKWVCKICGYIYDEDAGDPDNGISPGTKFEELPDDWVCPICGAPKSE FEKLEDMAKWVCKICGYIYDEDAGDPDNGISPGTKFEELPDDWVCPICGAPKSEFE KLED || ANIKLSVQMKLFKRHLKWKIIVKLN DGRELSLD

Figure 1: Sequence of Cleaved RdRd -Anginex: amino acids in red color represent the RdRd tag and the amino acids in black color represent the Anginex.

Results

Small scale expression of RdRd-Anginex

To express RdRd-Anginex on a larger scale, the plasmid was transformed into *E. coli* strain BL21* (DE3). With a successful transformation, colonies were picked and expressed on a small scale. The harvested cells were sonicated, and samples were run on an SDS-PAGE to verify the molecular weight. The supernatant shows the expression of RdRd-Anginex (Figure 2).

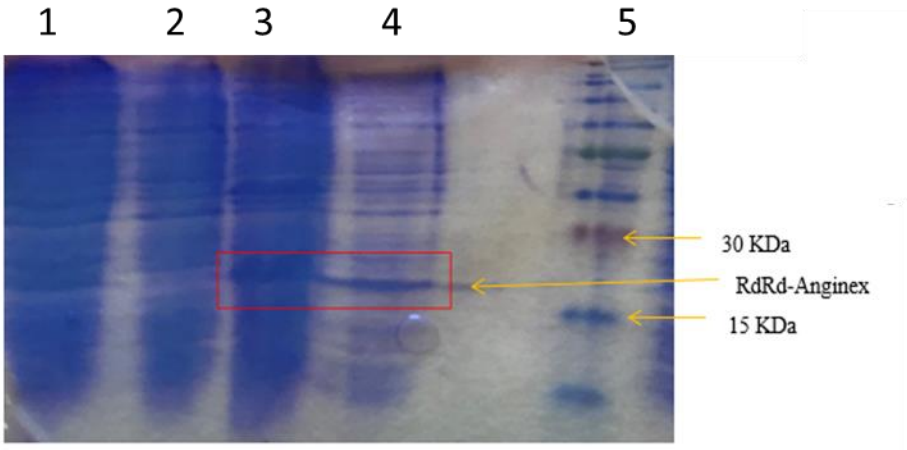

Figure 2: SDS-PAGE of small-scale expression of RdRd-Anginex: Lane 1, Pellet; Lane 2, Uninduced; Lane 3- 4, Supernatant of RdRd-Anginex; Lane-5, Protein ladder

Purification of RdRd-Anginex on Nickel Affinity Chromatography

RdRd-Anginex was purified using Nickel-Affinity Chromatography. The SDS-PAGE analysis shown in (Figure 3) illustrates that RdRd-Anginex elutes in the 50 mM Imidazole, 100 mM Imidazole, 250 mM Imidazole, 500 mM Imidazole, and Urea fractions as shown in lanes 2 to 6. The bands that are highlighted in lanes 2 to 6 indicate the presence of RdRd-Anginex at a molecular weight around 17 kDa. RdRd-Anginex is significantly eluted in the 100 mM Imidazole fraction due to it having the largest band in lane 4. However, in all lanes, there was evidence of higher and lower molecular weight contaminants (37KDa and 6KDa) present.

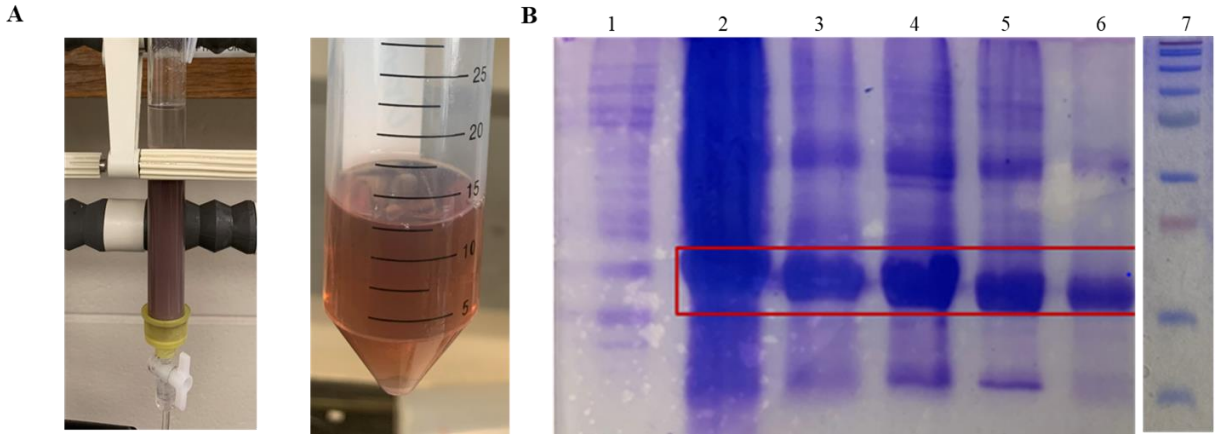

Figure 3: Purification of RdRd-Anginex from Nickel Sepharose chromatography. Panel A: RdRd-Anginex on Nickel Sepharose column, Panel B: Fractions eluted from purification of RdRd-Anginex on Nickel Sepharose. Lane-1, Flow through; Lane-2, 20Mm imidazole; Lane-3, 50mM imidazole; Lane-4, 100mM imidazole; Lane-5, 250mM imidazole; Lane-6, 500Mm imidazole; Lane-7, protein ladder.

To obtain pure RdRd-Anginex protein, the flowthrough from each imidazole fraction was subjected to a buffer exchange using ultrafiltration. RdRd-Anginex following buffer exchange resulted in approximately 90% purity level for the 500mM imidazole fraction (Figure 4).

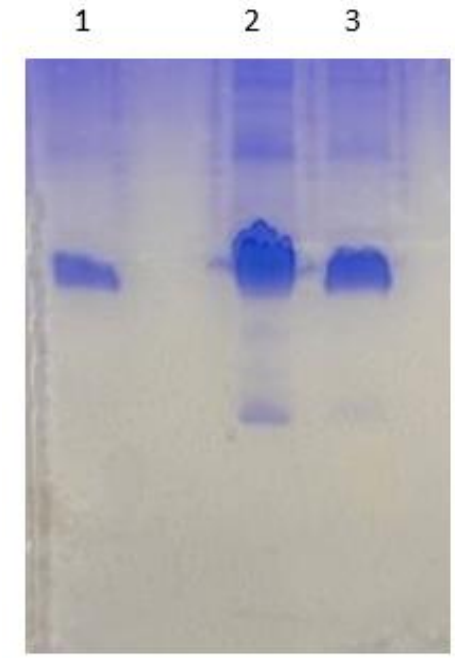

Figure 4: SDS-PAGE analysis after ultrafiltration of RdRd-Anginex: Lane 1, Buffer exchanged 500mM imidazole fraction; Lane 2, Buffer exchanged 100mM imidazole fraction; Lane 3, Buffer exchanged 250mM imidazole fraction.

Enterokinase Cleavage

Typically, after the complete cleavage of a protein, two bands are observed on an SDS-PAGE; the target protein and the tag used. In the case of RdRd-Anginex, one protein band is observed after complete cleavage (Figure 5). Anginex, the target protein is 4KDa, and RdRd from previous work shows that it moves as approximately 5KDa on an SDS-PAGE. The one band observed on the gel might be Anginex and RdRd moving at the same molecular weight. MALDI-TOF MS on the cleavage products indicates the presence of both target proteins, Anginex and RdRd. The Silver stain was also employed to visualize the Anginex protein band (Figure 6). The results show that Anginex protein band appears as expected on an SDS-PAGE (Figure 6). Upon expressing RdRd-Anginex in 1 L of media, a yield of 2mg of Anginex protein was obtained.

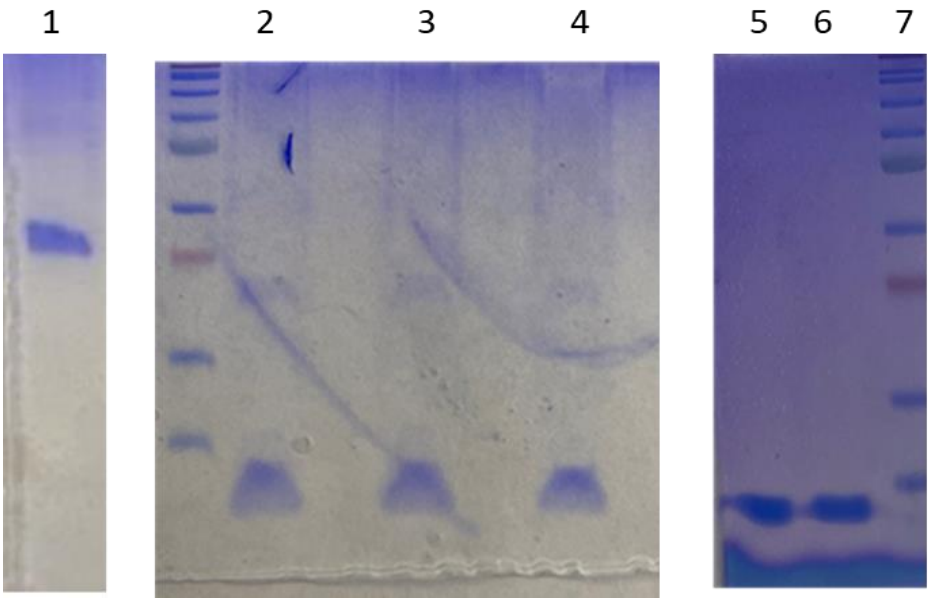

Figure 5: Cleavage of RdRd-Anginex. Lane 1, Buffer exchanged of 500mM imidazole fraction. Lane 2, Cleavage product; Lane 3, Anginex; Lane 4; RdRd; Lane 5-6, Buffer Exchanged Anginex; Lane 7, Protein ladder.

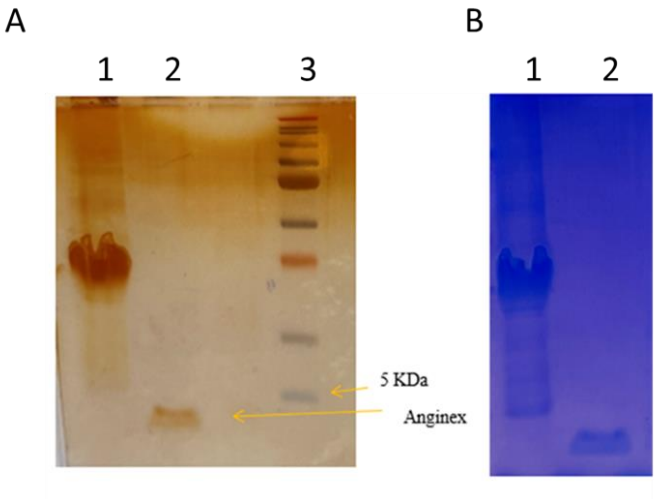

Figure 6: SDS-PAGE analysis gel of eluted Anginex: Panel A: Silver stain Panel B: Coomassie Blue Stain Lane 1, RdRd-Anginex; lane 2, Anginex; lane 3, protein ladder.

Mass Spectroscopy of RdRd-Anginex

Due to the anomalous mobility of the RdRd-Anginex cleavage product on SDS-PAGE, the protein was analyzed using Matrix-assisted laser desorption-ionization time of flight mass spectrometry (MALDI-TOF MS) to confirm the molecular weight. MALDI-TOF MS is an analytical technique in which particles are ionized, separated according to their mass-to-charge ratio, and measured by determining the time it takes for the ions to travel to a detector at the end of a time-of-flight tube. (12) RdRd's molecular was confirmed to be approximately 14KDa and Anginex to be (4KDa) (Figure 7).

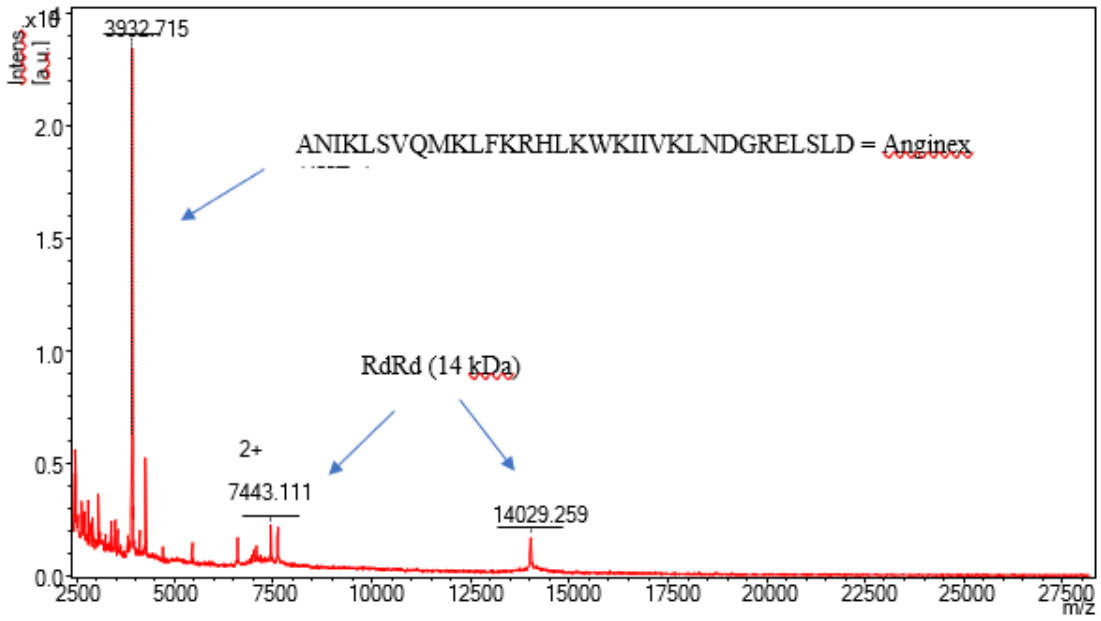

Figure 7: Mass Spectroscopy Spectrum of Cleaved RdRd-Anginex shows the molecular weight of Anginex and the molecular weight of RdRd

Conclusion

In summary, recombinant Anginex with Rubredoxin tag was expressed and purified efficiently. The purification of RdRd-Anginex using affinity chromatography allows for easy visualization during and after the purification process due to the red color of RdRd. Also, the RdRd tag on Anginex allowed higher stability and expression of Anginex throughout the large-scale

expression and purification process. Enterokinase was found to cleave RdRd and Anginex effectively after about 48 to 60 hours without degradation. Future studies will reveal the anti-angiogenic activity of this recombinant Anginex.

Acknowledgment

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