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Design of Antimicrobial Peptide Based on the
Heparin Binding Segment of FGF-1

*An Honors Thesis submitted in partial fulfillment of the
requirements for Honors Studies in Biochemistry*

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Academic School Year: 2014-2015
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Index:

<i>Abstract</i> _____	3
<i>Introduction</i> _____	4
<i>Materials/Experimental Methods</i> _____	15
<i>Results and Discussion</i> _____	20
<i>Conclusions</i> _____	30
<i>Acknowledgements</i> _____	33
<i>References</i> _____	34

Abstract

Fibroblast Growth Factors (FGFs) are heparin-binding proteins known for their involvement in various biological processes such as cell differentiation and wound healing.¹ The heparin binding site of FGF-1 displays a unique stretch of positive amino acid sequence and facilitates a very strong binding to negatively charged heparin due to the formation of electrostatic interaction. The aim of this study is to analyze and modify the novel antimicrobial peptide sequence (GST-HB) designed based on the heparin-binding region of FGF-1 as well as other polycationic microbial sequences. These aspects will be examined using various experimental techniques including overexpression of GST-HB, purification using one-step affinity column chromatography, and bacterial assays. The binding affinity of FGF's to heparin is a crucial component to bacterial infection pathways. Therefore, antimicrobial applications will be determined for the recombinant GST-HB peptide for future medical treatments of bacterial infections.

Introduction

Glycosaminoglycans and Heparin Analogs:

Glycosaminoglycans (GAGs) play a critical role in the pathophysiology of diseases including diabetes, atherosclerosis, and metastasis.^{8,11} GAGs localize proteins and enzymes at their site of action to facilitate their physiological functions and in some cases prevent their proteolytic degradation.⁸ They also regulate protein secretion and gene expression in certain tissues by mechanisms involving events in the membrane and nucleus.^{8,11}

Heparin sulfate (HS), an analog of heparin, is classified as a glycosaminoglycan as well. This molecule is present in the cell surface membranes and is essential to the extracellular matrix.⁶ HS has a structure similar to that of heparin consisting predominantly of a repeating disaccharide comprised of β -D-glucuronic acid and N-acetyl- α -D-glucosamine.⁷ The D-glucuronic acid moieties are not as fully sulfated resulting in less of a negative charge than heparin.^{7,8} According to a study done by F. Zhang, Z. Zhang, X. Lin, et al., the FGF-Heparin complex is more stable than the FGF-HS complex since heparin decassacharide and the 6-desulfated heparin decassaccharide more strongly inhibited binding of the FGF-FGFR complex to heparin.⁷ Sucrose octasulfate (SOS) is another structural analog of heparin and has shown to mimic heparin by supporting the FGF-mediated cell proliferation activity.^{14,17} Both heparin and SOS are shown to promote osteocalcin expression and promote complete closure of the cranial sutures.^{14,16} SOS activates the FGF receptor by binding to the heparin binding sites in both FGF and the D2 domain of the FGFR.^{14,16} Charge

interactions play a dominant role in the D2-SOS interaction because the SOS binds to positively charged residues in the D2 domain.¹⁴ Since the SOS-D2 domain interaction is in the same range as that reported for heparin binding, the D2 domain largely contributes the heparin-binding site.¹⁴ The crystal structure of the FGF2-FGFR1-SOS complex at 2.6 Angstrom resolution reveals a symmetric assemblage of two 1:1:1:FGF2-FGFR1-SOS ternary complexes.¹⁵ SOS binds to FGF and FGFR which increases FGF-FGFR affinity and interacts with adjoining FGFRs which promotes protein-protein interactions that stabilize dimerization.¹⁵ Therefore, SOS can promote FGF signaling by imitating the role of heparin in enhancing FGF-FGFR affinity and receptor dimerization.^{14,15}

Applications and Significance of Heparin:

Heparin is found in mast cells and is isolated for clinical uses from hog mucosa or beef lung. It is released from mast cells in response to an external signal. Clinically, it is commonly used as an anticoagulant.⁶

A major characteristic of heparin is that it is classified as a linear sulfated glycosaminoglycan (GAG). It consists predominantly of a repeating disaccharide comprised of an uronic acid residue (either a D-glucuronic acid or L-iduronic acid) and a D-glucosamine, which is either N-sulfated or N-acetylated.⁶ Alpha 1,4 linkages connect the uronic acid residue and D-glucosamine in the structure of heparin.⁶ These sulfate groups impart a strong negative charge on the heparin chain.⁸ The content of this compound consists of multiple residues that contain sulfur groups, which play important roles in the regulation of heparin specificity in cellular proteins including various growth and differentiation factors, extracellular matrix (ECM) components, protease inhibitors,

protease, lipoprotein lipase, and various pathogens.⁶ Each disaccharide unit contains a sulfur group at the 2-amino position on the uronic acid residue and the 6-amino, 3-amino, and N-amino groups of the glucosamine residue.^{6,9,10} The interactions of these sulfur groups with other proteins play a pivotal role in various patho-physiological phenomena through electrostatic interactions.

The extracellular matrix plays a central role in the control of cell proliferation, differentiation, and migration by mediating cell adhesions and communication.^{6,11} Therefore, the extracellular matrix contains heparin binding proteins that help regulate the physiological processes that heparin contributes to. Heparin binding proteins are produced by diverse cell types and are found in a variety of tissues. These groups include the proteins of the circulatory system involved in coagulation, many growth factors, and proteins involved in lipid metabolism.^{6,11} Heparin binding proteins are characterized by a certain distribution of basic amino acids, especially lysines and arginines along a relatively short amino acid sequence.⁶ There are many proteins that contain such sequences which illustrates the versatile interactions that heparin has with other proteins.⁶ Heparin adopts a helical structure and has torsion angles associated with the intermonosaccharide glycosidic bonds and the pyranose ring of α -iduronic acid.¹² The pyranose ring of the α -iduronic acid can potentially adopt many conformations. The conformational flexibility of this ring enhances the specificity of heparin when binding to other protein.¹² Furthermore, the ability of this iduronic acid to adopt multiple ring conformations enhances the formation of a kink, which is formed when heparin binds to other proteins such as fibroblast growth factors.^{12,13} This kink

provides optimal ionic and van der Waals contact with the protein that heparin is binding to which influences the specificity of heparin.^{12,13} The overall heparin helical structure is maintained when bound to another molecule, but local conformational changes in the heparin structure occur due to the backbone torsion angles formed from the kink.¹² The spatial distribution of basic amino acids in the heparin-binding loops influences the distribution of sulfate groups in the interacting portion of the heparin chain, and thus provide the best ionic and surface interactions for heparin binding.¹²

Need for Purification of Heparin:

In 2008, over-sulfated chondroitin sulfate (OSCS) and dextran sulfate (DS), two compounds similar to heparin, were found in batches of heparin that killed hundreds of patients.^{2,5} The similarities between OSCS, DS, and heparin made it impossible to purify heparin. Heparin and OSCS are alike in that both are members of a family called glycosaminoglycans and have similar molecular weights. OSCS has harmful effects on the body because it activates the kinin-kallikrein pathway, which eventually creates bradykinin.^{3,4} Bradykinin leads to low blood pressure by inducing a physiological response that dilates the blood vessels. Upon exposure to the contaminated heparin, other symptoms included shortness of breath, nausea, rashes, and tachycardia.^{2,5} These contaminated shipments of heparin came from the Changzho SPL plant in China and affected over 10 countries and an extensive recall was required.⁴

Fibroblast Growth Factors (FGFs), Fibroblast Growth Factor Receptors, and Heparin:

The fibroblast growth factor family includes 23 structurally related proteins with a core region of approximately 130 residues that consist of twelve antiparallel β -strands.^{18,19} In development, FGF's are required for embryogenesis and organogenesis,

which is the production and development of the organs.¹⁴ FGF's continue to regulate tissue homeostasis in the adult and play important roles in angiogenesis, differentiation, tissue repair, wound healing and cholesterol metabolism.^{14,20}

FGF's perform their diverse functions by binding and activating cell surface FGF receptors (FGFR). FGF-FGFR binding specificity is essential for the regulation of FGF signaling and is determined by differences in the protein's primary sequence.^{7,8} Receptor dimerization in FGF signaling requires the presence of the highly sulfated heparin polysaccharide chains and can cause a wide spectrum of human pathological conditions including blood coagulation and cancer.⁸ Signaling begins with the formation of a ternary complex of FGF, FGFR, and heparin.⁷ Heparin is known to bind tightly to FGFs since it has a dissociation constant in the nanomolar range.⁷ The strong binding of FGFs to heparin is important physiologically because it deters degradation of FGFs from acid and heat.^{8,21}

There are four known FGF receptors (FGFRs), FGFR-1 through FGFR-4.⁸ The prototypical FGFR consists of an extracellular domain, a single-pass transmembrane helix, and a cytoplasmic tyrosine kinase domain.¹⁴ The FGFRs share between 55% and 72% homology at the protein level. FGF signaling is triggered by binding of the ligand (FGF) to the extracellular domain of the FGFR, which causes a phosphotyrosine-induced conformational change in their target proteins to activate various catalytic activities.⁸ The extracellular domain consists of three immunoglobulin-like domains (D1-D3) and an acid box (a continuous stretch of four to eight acidic amino acids) placed between the D1 domain and the D2 domain.^{14,22} The first Ig-like domain is thought to play a role in

receptor autoinhibition. Domains I and II constitute the FGF ligand-binding site.¹¹ FGF induced dimerization of the receptor is a mandatory step in FGF-induced signaling. Binding of FGF to the extracellular domain of the FGFR leads to receptor dimerization and juxtaposition of the tyrosine residues in the cytoplasmic domain.⁸ [The receptors then become capable of phosphorylating specific tyrosine residues on their own and each other's cytoplasmic tails.^{8,23} Phosphorylation of seven tyrosine residues in the receptor site is an essential step in activating the downstream signaling process to induce physiological processes.⁸ Such signaling pathways include the PLC λ , Src, Crk-mediated, and SNT-1/FRS2 which are involved in cytoskeleton alteration, linking FGFR signaling to a protein that binds actin, mitogenesis, and growth factor induced cell-cycle progression respectively.^{8,24}

As mentioned before, different FGFs can induce a variety of responses in the body since they can bind to a range of target cells that include but are not limited to fibroblasts, endothelial cells and keratinocytes.⁸ FGFs have specific receptor sites on the surface of their respective target cells. Therefore, cells must express different forms of the FGF receptor. This is accomplished through the expression of splice variants of a given FGFR gene or by expression of different FGFR genes themselves.⁸ Alternate splicing of the FGFR gene codes for a variety of different receptor protein isoforms made possible because of the structure of the respective genes.⁸ A 5' non-translated sequence, a hydrophobic signal sequence, the IgI and IgII sequences separated by an acid box, the 5' end of the IgIII sequence followed by three possible 3' ends of IgIII that are due to alternative splicing, the transmembrane domain, and finally the kinase

domain are found in the mRNA that codes for the FGFR prior to splicing.^{8,25} Different exon usage allows the translation of proteins which may be prematurely truncated, lack Ig-like domains, or utilize different coding regions for the same Ig-like domains.⁸ This results in different expression of the mRNA for FGFRs and thus altered specificity for different FGFs.

Even though the IgIII domains (IgIIIa, IgIIIb, and IgIIIc) that play a pivotal role in FGF binding specificity are more homologous between genes than between each other, there are other receptor domains besides IgIII that affect binding specificity.^{8,26} Therefore, diversity in FGF signaling can be achieved through analogous splice variants of different genes as well.

FGF Signaling in Diseases:

Human cancers develop through a multi-stage process that transforms normal cells into malignant cells by accumulating a number of genetic changes.¹¹ Because FGFs activate many signal transduction cascades, various genetic programs are activated which stimulate cell growth, regulate transcription factors, and inhibit pathways of cell death.²⁷ Therefore, all components of this pathway, from the polypeptide growth factors to the transcription factors are potential oncoproteins.²⁷ Decreased regulation at any of these cascades controlled by FGF signaling promotes cell growth beyond control and causes cancer.^{27,29} FGFs and FGFRs seem to stimulate several steps of cancer progression including cancer cell proliferation and survival, as well as invasion, metastasis, and angiogenesis.⁸ There are no documented mutations in FGFs themselves so the most logical mechanism that FGFs contribute to unregulated cell growth is by

overexpression.²⁷ This most likely occurs through the overexpression of FGFs by the tumor cells themselves, secretion of FGFs by the stromal cells in response to a signal from the tumor cells, and secretion of FGFs by the tumor in response to a signal from non-transformed cells.²⁷ Examples of these mechanisms are illustrated by FGF-2 and FGF-5. FGF-2 has shown to be associated with the degree of malignancy and vascularity in human gliomas, which is expected since FGF-2 acts as an autocrine growth factor on the tumors and promotes angiogenesis in the surrounding stroma.²⁷ FGF-5 is secreted as a paracrine growth factor by the non-transformed cells in the tumor stroma in response to signaling factors present in the tumor. Another possibility for deregulation of FGF signaling in cancer could be due to the mobilization of FGFs from the extracellular matrix.²⁷ Two models for the release of FGFs include enzymatic cleavage of extracellular matrix components by proteases or heparanases, an enzyme that acts to degrade heparin, or by binding to carrier proteins, which deliver FGFs to their receptors.²⁷ Studies have investigated the release of soluble FGFs from the ECM by digestion of the glycosaminoglycan portion of heparin-like glycosaminoglycans (HLGAGs) through the activity of heparanases. HLGAGs are required components of the activated receptor complex along with FGFs. FGF binding proteins present FGFs at the cell surface where it interacts with HLGAGs and in turn present FGF to the FGFR to activate various signal cascades. The mammalian gene for heparanase is expressed at the mRNA and protein levels in metastatic human and rat cell lines.²⁷ The ability of heparanases to release bound stores of FGFs triggers angiogenesis, which is consistent with the observed role of

heparanases in promoting metastasis.²⁷ Therefore, regulating expression of heparanases some tumors may be able to mobilize FGFs from the ECM.

With regards to pathogen infections, growth factors are incorporated into the genome of infectious pathogens because they provide an advantage of allowing the infectious agent to bind to the host cell and gain access to the surface proteins that facilitate infection.²⁸ Furthermore, pathogens utilize GAG-mediated processes such as chemokine signaling. It is known that GAG's play a role in the herpes simplex virus (HSV) and human immunodeficiency virus (HIV) based on the inhibitory effect of heparin.^{28,30} Heparin binding FGFs are found in the viral envelope, which suggests viral infection is linked to growth factor signaling by mimicking the heparin-binding domain.²⁸ GAG-binding proteins are important in adhesion and invasion when dealing with bacterial pathogenesis.²⁸ Diverse bacteria bind to heparin on the surface of cells. For example, Chlamydia attaches to the host and is mediated by GAG bridges between protein receptors on both bacteria and host cells.^{28,31} Thus, heparin controls invasion of bacteria into the host cell.^{28,31} Therefore, heparin represents a new antibiotic approach because it facilitates bacteria's mechanism of entering into the host cell.

Bacteria are protected from the external environment by their complex outer membrane structures. The membrane has an asymmetric lipid bilayer with negatively charged lipopolysaccharide (LPS) molecules localized on the outer leaflet and is stabilized by the presence of cations.³² Many studies have shown that polycationic molecules can damage the outer membranes of bacteria and enhance bacterial susceptibility to hydrophobic substances such as antibiotics and lysozyme.³² A known

compound that utilizes this mechanism is Polymyxin. Polymyxin is a polycationic decapeptide antibiotic with a fatty acid tail that contains five positively charged groups and no negatively charged groups.³³ Its ultimate lethal target is generally thought to be the cytoplasmic membrane to which it will become bound to phospholipids and therefore destroy the cytoplasmic membrane of the bacteria.³³ Polymyxin gains access through the disruption of the outer membrane by causing extensive alterations due the polycationic nature of its structure by binding to the anionic core-components of LPS molecules.^{32,33} Therefore, polycationic molecules have proven to be effective methods of treatment for bacterial infections.

A novel heparin-binding affinity tag was developed by Dr. Kumar to exhibit a greater binding affinity for heparin and thus optimize purification. Applications of this enhanced heparin binding will be critical to this project since it is known that heparin plays a crucial role in bacterial infection pathways. The polycationic nature of the HB portion of the GST-HB peptide developed by Dr. Kumar is due to the presence of many arginine and lysine amino acids. Therefore it is expected to exhibit antimicrobial properties by binding to heparin in a fashion much like FGF-1. The following amino acid sequence of FGF-1 highlights the heparin binding site region in bold:

MAEGEITTFALTEKFNLPNGYKKPKLLYSNGGHFLRILPDGTVDGTRDRSDQHILQLLSAESVGEV
YIKSTETGQYLAMDTDGLLYGSQTPNEECLFLERLEENHYNTYISKKHAENWVGL**KKNGSCKRGP**
RTHYGQKAILFLPLPVSSD. This sequence of amino acids creates a positive charge for the heparin-binding domain of FGF-1, which interacts with the negatively charged heparin, a polysaccharide that is crucial for the activity and function of various proteins. The

polyanionic surface of heparin binding to this polycationic site of FGF-1 is critical for FGF interactions. Therefore, the polycationic nature of the HB peptide is expected to exhibit a great affinity for heparin and thus inhibit the FGF-FGFR interactions that are essential for facilitating bacterial attachment and invasion into cells.

Materials and Methods

Materials include *Escheria coli* BL-21 cell, pGEX-KG-Hep expression vector, GST-HB glycerol stock, isopropyl B-D-1-thiogalactopyranoside (IPTG), terrific broth (TB), 100% glycerol, ampicillin, phosphate buffered saline (PBS), L-reduced glutathione 99%, GSH Sepharose resin, 20% ethanol, 8M urea, thrombin, 10KDa Macrosep Advance Centrifugal Device, 30% acrylamide, sodium dodecyl sulfate (SDS), ammonium persulfate (APS), TEMED, Coomassie Blue, methanol, 2-B-mercaptoethanol, bromophenol blue, hydrochloric acid, DH5 α host cells, egg white lysozyme, Luria-Bertani (LB) agar, LB broth, sterile Whatman paper disks, high definition Sony camera.

Construction of Novel Antimicrobial Peptide GST-HB:

The nucleotide sequence corresponding to HB amino acid sequence was cloned downstream of GST sequence in order to be further expressed at high yields in bacterial systems. The recombinant peptide was cleaved from the fusion partner, GST, by using a thrombin cleavage site, LVPRGS, incorporated at the C-terminal end of the peptide between GST and HB. Critical to the construction of the recombinant peptide was a vector developed by Dr. Srinivas Jayanthi. This vector carries the operon that contains the necessary genetic information to make copies of mRNA corresponding to the nucleotide sequence of the peptide. Furthermore, it contains genes that have antibiotic resistance, which is essential for allowing GST-HB to be expressed. The extensive details of this vector are detailed in Figure 1. Since only the HB peptide is thought to have antimicrobial properties, its separation from GST, using thrombin, is crucial for further antimicrobial studies.



The structure above shows a basic layout of the peptide. The GST portion is represented in blue. The orange portion represents the thrombin cleavage site followed by HB represented in green.

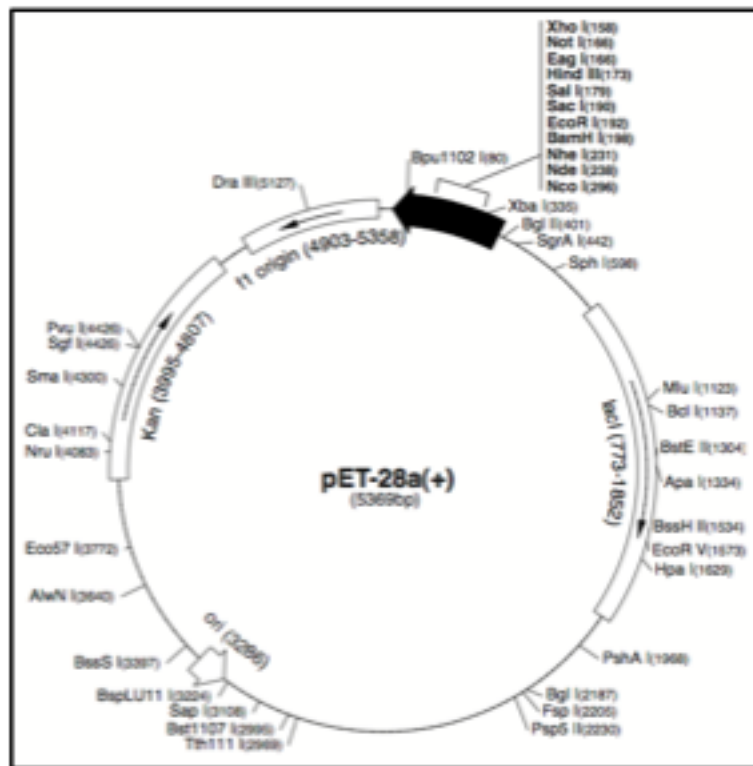


Figure 23. Vector map of pET-28a used for cloning HCW

Figure 1: The extensive work done by Dr. Srinivas Jayanthi resulted in the development of pET-28a: the vector necessary to clone the recombinant peptide.

Overexpression of GST-HB:

GST-HB will then be overexpressed in BL21 cells using already established experimental methods. Glycerol stocks of *E. Coli* cells transformed in the pGEX-KG-Hep vector were inoculated for 14-16 hours in terrific broth with ampicillin (1uL/mL of terrific broth) and grown overnight at 37 degrees Celsius and 250 rpm. After growing

overnight, 5% inoculum and ampicillin (still 1uL/mL) were added to autoclaved terrific broth. This was then allowed to inoculate under the same conditions for 1-1.5 hours to allow for the optical density of the solution to reach an absorbance of 0.6-1.00 at 600 nm. Cultured flasks were then induced with 1mM (500 μ L) of isopropyl β -D-1-thiogalactopyranoside (IPTG) and allowed to incubate for another four hours. The culture was then centrifuged at 6000 rpm for 20 minutes to form bacterial pellets. The supernatant was discarded and the pellets were resuspended in approximately 50 mLs of 1xPBS buffer at pH 7.2. These samples were subjected to centrifugation again at 6000 rpm for 20 minutes. The supernatant was discarded and the pellets were stored at -20 degrees Celsius until further use. The overexpression of GST-HB was analyzed using 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Purification of GST-HB:

Affinity chromatography using GSH Sepharose was performed to purify the 1L GST-HB pellets created using the overexpression methods just described. The pellets made using the previously described overexpression method were resuspended in 30 mLs of 1xPBS buffer at pH of 7.2. While on ice, sonication of the sample was employed for 30 cycles at 10-second intervals in order to lyse the protein. After sonication, the sample was centrifuged at 19,000 rpm for 20 minutes and the supernatant was carefully loaded onto the GSH sepharose column at a flow rate of 1mL/min followed by washing with 1xPBS at pH 7.2. The eluent buffer, 10 mM glutathione, was then loaded onto the column at a flow rate of 1mL/min to elute GST-HB off the column. Fusion protein was collected at this step and then subjected to concentration at 3600 rpm and 4 degrees

Celsius using an Amicon concentrator (10Kda cutoff) until a final volume of approximately 2-3 mLs was reached. Fractions of collected protein eluents (supernatant, 10mM reduced glutathione, and 8M urea) were analyzed using a 15% SDS-PAGE gel that was stained with Coomassie Blue to determine the success of the purification. The pure GST peptide will then be used for further experiments.

Optimal Thrombin Cleavage for GST-HB:

The purified fusion protein (GST-HB) was then subjected to cleavage by thrombin. The concentration of the fusion protein was estimated based on the extinction coefficient value of the protein and its absorbance measured using UV spectroscopy at 280 nm. One Unit of thrombin was added for every 250 µg of fusion protein. The mixture of thrombin and fusion protein was allowed to incubate for 20 hours at room temperature. Heat treatment at 65°C for 20 minutes yielded the pure HB in the supernatant. After that, the supernatant from the heat treatment then underwent desalting using 80% isopropanol. After isopropanol was added, the sample was incubated at -20 degrees Celsius for 3 hours. The mixture was centrifuged at 13,000 RPM for 10 minutes and the supernatant was removed to collect the cleaved peptide. The resulting pellet was dried to remove any trace of isopropanol.

Antimicrobial Activity:

Antimicrobial activity of GST peptide was measured and assessed on a gram-positive (BD99) and gram-negative (DH5α) bacterial strain. An already established method created by Vogt and Bechinger was used to quantify the inhibition of bacterial growth by the GST peptide to determine its MIC value. Experimentation using different

concentrations of lysozyme, fusion protein, and cleaved protein on both strains of bacteria was carried out using triplicate measures. In total, each assay consisted of two plates with disks containing the samples being tested and a disk containing ampicillin. The ampicillin served as the positive control for the experiment. A third plate without any disks plated served as an experimental control.

A lysozyme assay was utilized for standardization. A stock of 20mM lysozyme in sterile 1xPBS solution was created and underwent a serial dilution to create solutions with concentrations of 10mM, 5mM, and 1mM lysozyme respectively. Each strain of bacteria was propagated and allowed to grow for 14-16 hours in 10mLs of Luria-Bertani broth (LB) broth. Twenty mLs of Luria-Bertani (LB) agar were poured into each sterile petri plate and allowed to solidify. Once solidified, 100 μ L of each bacterial strain was added onto their respective plates and spread using a sterile flamed instrument. Ten microliters of each lysozyme concentration as well as a diluted sample of ampicillin (1 μ L ampicillin in 99 μ L of sterile 1xPBS) were loaded onto their individual sterile disk and plated in their corresponding labeled section that was previously outlined on the plate. The three plates for each complete assay were incubated at 37 degrees Celsius for at least 15 hours. After this time period, the plates were checked for zones of inhibition on the gram positive and gram negative strains of bacteria.

Solutions containing 1mM, 0.5mM, and 0.1 mM concentrations of fusion protein and cleaved peptide were created using sterile 1xPBS pH 7.2. These were tested on the DH5 α and BD99 cells using the same method and triplicate experimentation previously described for the lysozyme assay.

Results and Discussion

Overexpression and Purification of GST-HB:

The overexpression of GST-HB was achieved after adding 500 μ L (1mM) of IPTG to the respective cultured flasks. A sample of the culture was taken before adding IPTG and after adding IPTG to determine the success of overexpression. A strong band at 30 kDa indicates a positive overexpression and is illustrated in Figure 1 using SDS-PAGE techniques.

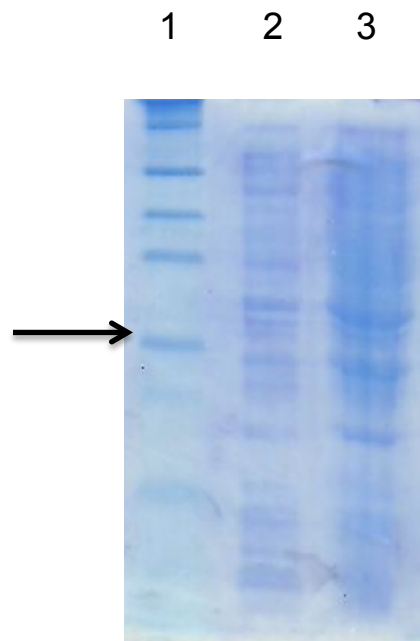


Figure 2: SDS-PAGE of GST-HB with a molecular weight of 30 kDa indicated by the black arrow. Lane 1 corresponds with the protein marker, lane 2 is the uninduced sample, and lane 3 is the induced sample. The correct band corresponding to GST-HB is seen on the gel.

The purification of pellets created in overexpression was accomplished by using GSH-Sepharose column chromatography. Eluents collected after loading the supernatant followed by washing with 1xPBS-pH 7.2, 10mM glutathione, and 8M urea were analyzed using SDS-PAGE techniques as well. A strong, pure band at 30 kDa indicates a successful purification of GST-HB as shown in Figure 2.

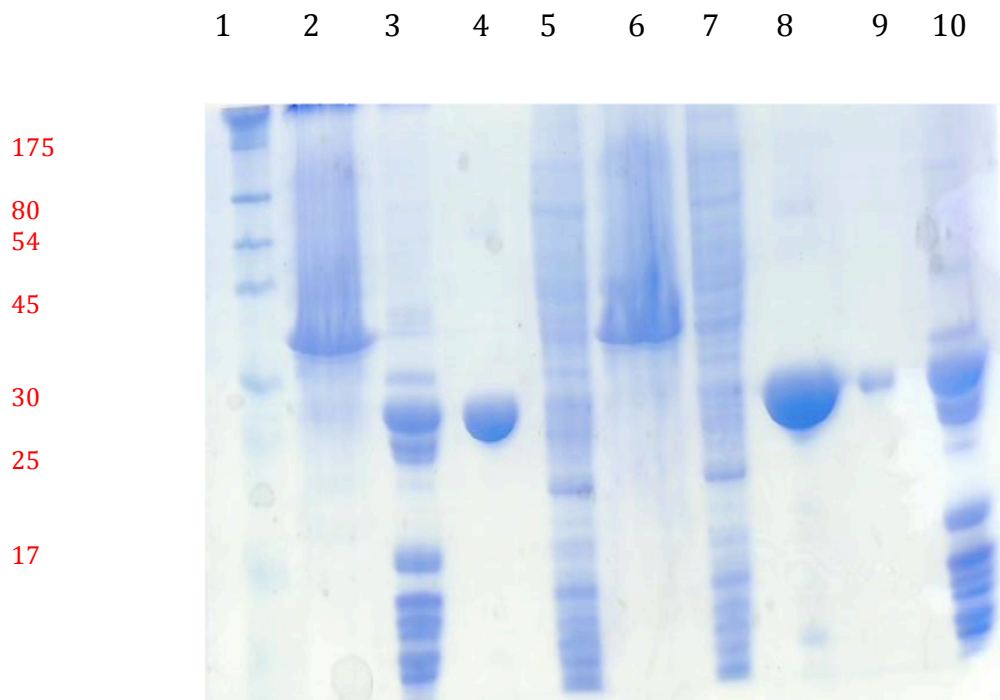


Figure 3: SDS-PAGE of GST-HB using GSH-Sepharose column. This particular gel indicates two replicates using purification methods. The buffer wash used was 1xPBS at a pH of 7.2. Lane 1- protein marker; Lane 2- pellet; Lane 3- 8M urea flow through; Lane 4- 10mM glutathione eluent (contains fusion protein); Lane 5- supernatant; Lane 6- pellet; Lane 7- supernatant; Lane 8- 10mM glutathione eluent (contains fusion protein); Lane 9- small sample from lane 8 drifted over into lane 9; Lane 10- 8M urea flow through.

Thrombin was utilized to cleave HB from GST. Cleavage of GST from the HB portion is critical because the HB portion is suspected to have antimicrobial properties. The GST portion of the protein is known to have a molecular weight of 26,000 Daltons

while the peptide has a molecular weight of 4,000 Daltons. Since the GST portion of the protein is significantly bigger than the HB peptide, a large pure band occurring at 26,000 Daltons and smaller, pure band at 4,000 Daltons indicates a successful thrombin cleavage. After heat treatment, the supernatant contains the pure HB, which is indicated by a single band at 4,000 Daltons.

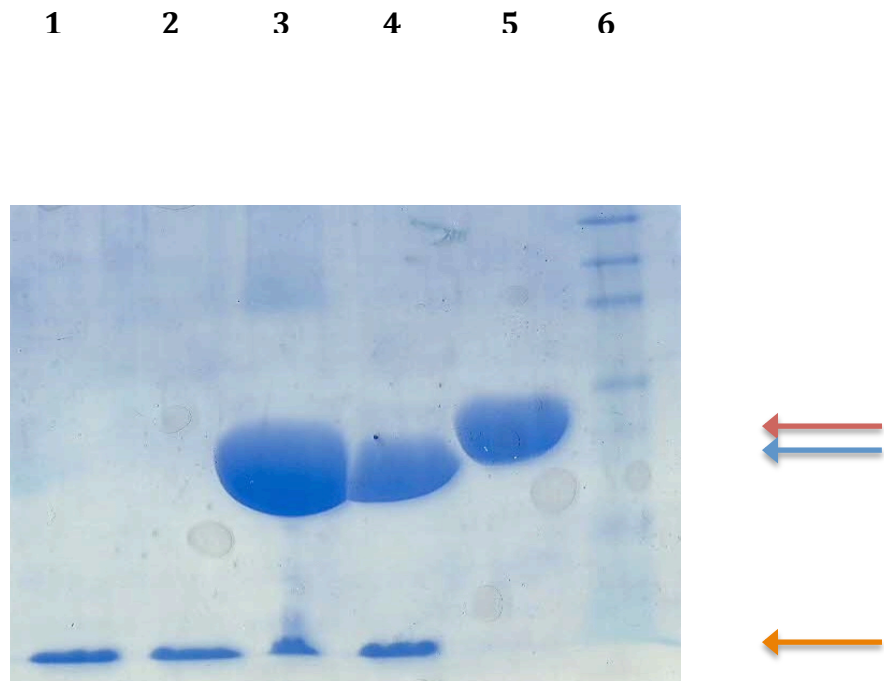


Figure 4: SDS-PAGE of GST-HB thrombin cleavage. Lane 1 corresponds to the heat supernatant collected from the thrombin cleavage containing the HB peptide. Lane 2 indicates heat supernatant containing peptide as well. Lane 1 and lane 2 are from two different purification samples. Lane 3 and lane 4 show successful cleavage of GST from the HB peptide using thrombin. Lane 5 is the GST-HB peptide before cleavage using thrombin. Lane 6 is the protein marker. The red, blue, and orange arrows correspond to the 30 kDa, 26 kDa, and 4 kDa bands on the protein marker respectively.

Gram Positive and Gram Negative Antimicrobial Assays:

Initial testing of the antimicrobial properties of the GST-HB peptide were accomplished by testing 1mM, 0.5 mM, and 0.1 mM concentrations of the fusion protein on both a gram positive (BD99) and gram negative (DH5 α) bacterial strains. After the purity of the peptide was determined by cleavage with thrombin, taking the absorbance of a sample dissolved in sterile 1xPBS and applying Beer's Law determined the pure peptide concentration. This concentration was used to create a stock solution of 1mM peptide, which then underwent a serial dilution to create 0.5 mM, 0.1 mM, and 0.05 mM solutions of peptide. Ten microliters of each concentration was plated using sterile Whatman disks and tested on a gram-positive (BD99) and gram-negative (DH5 α) bacterial strain. Each plate was incubated at 37 degrees Celsius for at least 12-15 hours before being checked for zones of inhibition. Finding a zone of inhibition indicates that there is indeed antimicrobial activity and therefore rendered successful results.

Similar experimental techniques were performed using triplicate measurements and concentrations of 1mM, 0.5 mM, 0.1 mM, and 0.05mM lysozyme. Lysozyme is known to be an effective inhibitor of the growth of bacterial cells by cleaving the peptidoglycan found in both gram-positive and gram-negative bacterial cell walls. Therefore, lysozyme was chosen for standardization of the peptide and fusion protein assays.

Any antibacterial activity documented due to the fusion protein or peptide shows promising applications towards inhibiting growth for various viruses and cancer cell lines.

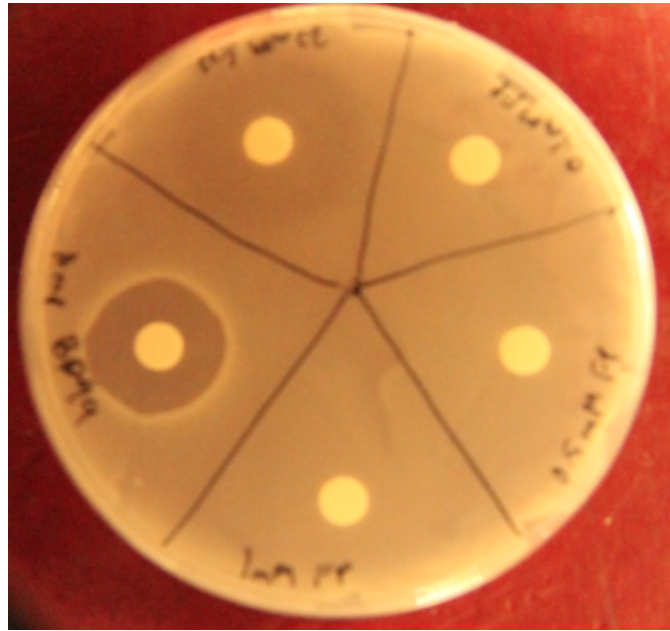


Figure 5: Result of testing 1mM, 0.5 mM, and 0.1 mM concentrations of the GST-HB fusion protein using BD99. Clear zones of inhibition are seen for ampicillin (positive control) and 20mM lysozyme. No clear zones of inhibition were recorded for the different concentrations of fusion protein.

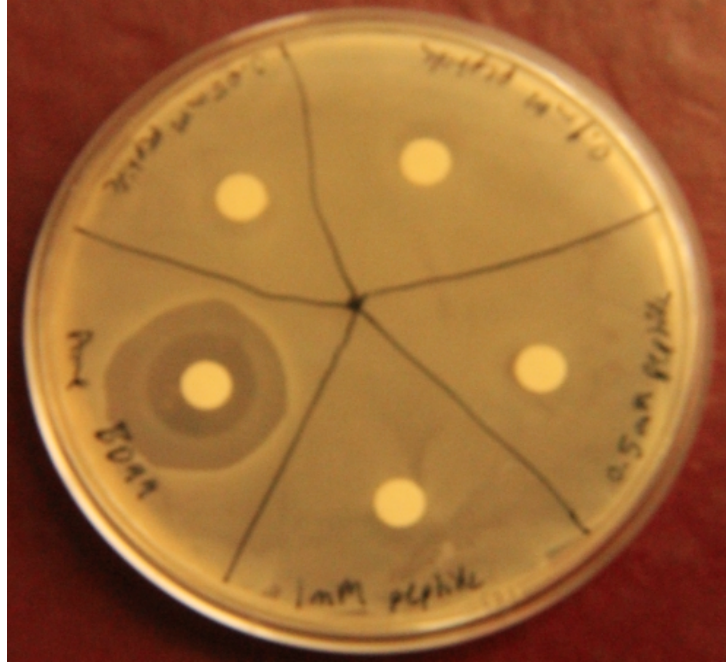


Figure 6: Result of testing 1mM, 0.5 mM, 0.1 mM, and 0.05mM concentrations of pure peptide. Zones of inhibition were recorded for all concentrations of the peptide and ampicillin (positive control).



Figure 7: Result of testing 1mM, 0.5 mM, 0.1mM, and 0.05 mM lysozyme concentrations. Clear zones of inhibition were recorded for the different concentrations of lysozyme indicating positive results. Ampicillin was used as the positive control.

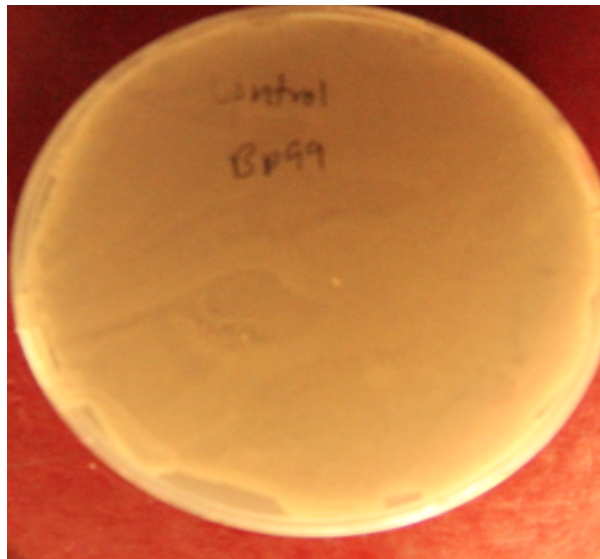


Figure 8: For each triplicate, a control plate of BD99 was plated to show a clean lawn of BD99 cells.



Figure 9: Result of testing 1mM, 0.5 mM, and 0.1 mM concentrations of the GST-HB fusion protein on DH5 α . A clear zone of inhibition is seen for ampicillin (positive control). Antimicrobial activity is seen for 20mM lysozyme as well (indicated by fuzzy zone surrounding disk). However, no clear zones of inhibition were recorded for the different concentrations of fusion protein.

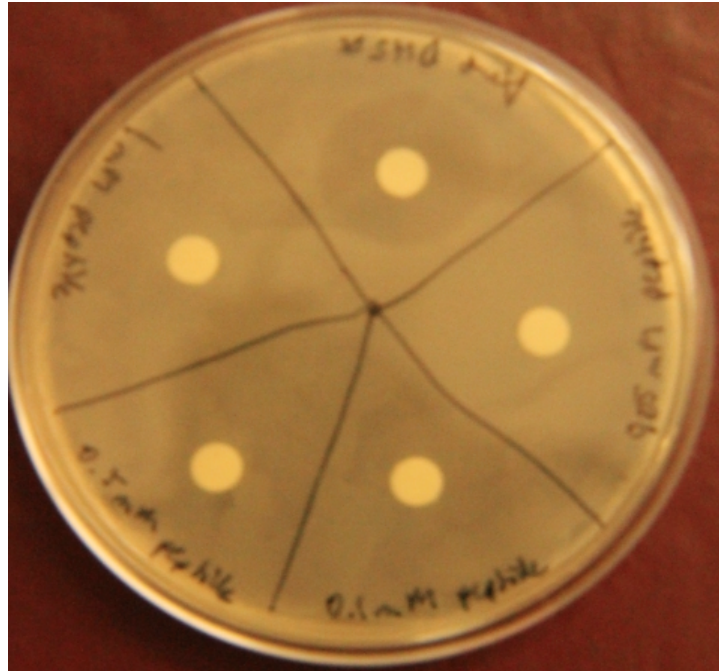


Figure 10: Result of testing 1mM, 0.5 mM, 0.1 mM, and 0.05mM concentrations of pure peptide on DH5 α . No zones of inhibition were recorded for all concentrations of the peptide. However, ampicillin was seen to have a strong zone of inhibition as expected.

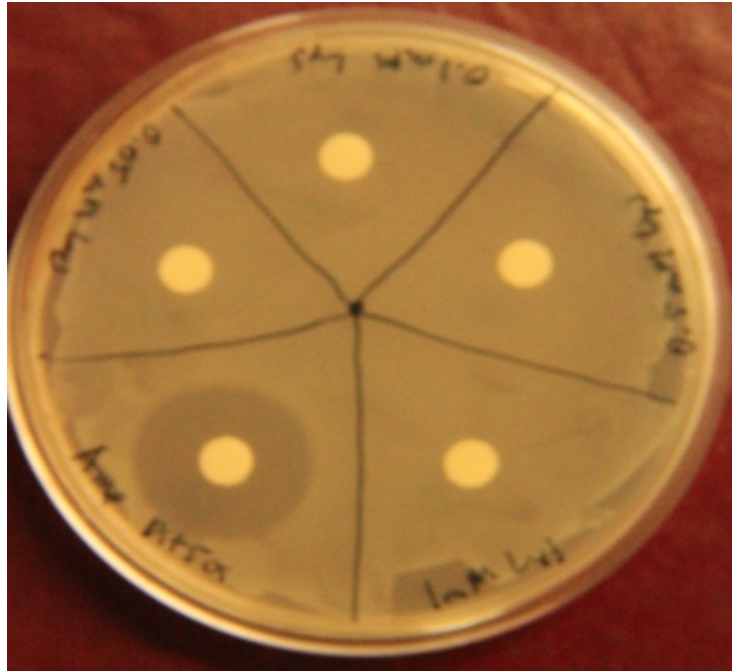


Figure 11: Result of testing 1mM, 0.5 mM, 0.1mM, and 0.05 mM lysozyme concentrations on DH5 α . No clear zones of inhibition were recorded for the different concentrations of lysozyme. Ampicillin was used as the positive control again and had a distinct zone of inhibition.

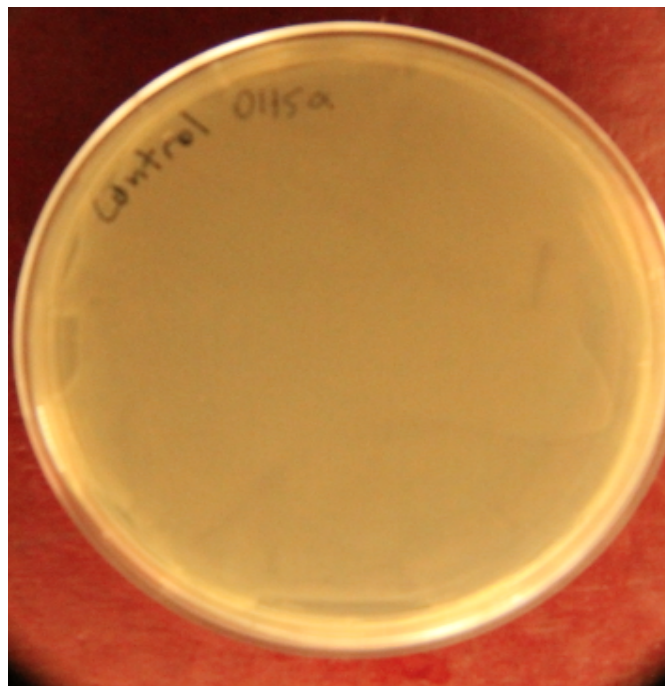


Figure 12: For each triplicate, a control plate of DH5 α was plated to show a clean lawn of gram-negative cells.

Conclusions

Because of the presence of a huge affinity tag like GST at the N-terminus of the HB peptide, there is a possibility for the interference of the GST tag on the activity of the peptide. This could have resulted in loss of anti-microbial activity. It makes sense that no zones of inhibition were recorded for the gram-positive or gram-negative strains of bacteria. There was not a significant amount of HB peptide to document antimicrobial activity since the GST portion of the peptide is six and half times more massive than the HB portion of the peptide. Therefore, the antimicrobial activity was essentially diluted due to proportions of GST to HB, which is the most likely cause for the failed documentation of zones of inhibition.

Lysozyme is a known inhibitor of bacterial growth and it exhibits antimicrobial properties based on its ability to cleave peptidoglycan in bacterial cell walls. Gram-negative bacteria have smaller amounts of peptidoglycan in their cell walls, whereas gram-positive bacteria are known to contain higher concentrations. Therefore, it was expected that lysozyme would have a lesser effect on the growth of gram-negative bacteria.

Comparing the lysozyme triplicate assay to that of the pure peptide tested on BD99 revealed that the zones of inhibition achieved for the 0.1 and 0.05 mM concentrations are smaller than that of the pure peptide. This indicates that the pure peptide is a more effective inhibitor of bacterial growth at these respective concentrations. The 0.5 mM concentration is equivalent to that of the pure peptide as well, but the 1 mM lysozyme zone of inhibition exceeds that of the pure peptide.

Despite the disparity at the 1 mM level, the pure peptide has established that, it inhibits gram-positive bacterial growth at smaller concentrations. The testing of the pure HB peptide yielded small zones of inhibition for all the concentrations resulting in successful experimentation with pure peptide on the gram-positive strain of bacteria. However, the zones of inhibition appeared to be larger for the smaller concentrations of pure peptide, which was contrary to the expected results.

Since the antimicrobial affects of the HB portion of the peptide depend on the ability of the peptide to disperse into the surround bacterial lawn, it makes sense that the smaller concentration of the peptide would exhibit better zones of inhibition since the lower concentration of peptide could exhibit a greater mobility factor. From the consistent results achieved from the triplicate assays, it can be inferred that the peptide could reach maximum antimicrobial activity at a certain concentration. However, the positive results of this assay indicate a degree of effectiveness with regards to this gram-positive bacterial strain.

The lysozyme assay, pure peptide, and fusion protein triplicate assays did not exhibit zones of inhibition when tested on *E.coli* DH5 α . Since the 20 mM concentration of lysozyme exhibited minimal amounts of antimicrobial activity, further experimentation with higher concentrations of pure peptide and fusion protein could expect low activity up until this concentration as well. Since the amount of fusion protein collected from each purification is relatively small, the further testing using concentrations at or above 20 mM would take significant amounts of materials, time, and effort beyond the scope of this project. However, the positive results obtained from

testing the peptide on gram-positive strains of bacteria indicate further applications and experimentation with this peptide are worth pursuing.

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