HPLC in the Purification of Antimicrobial Peptides from *Andrias davidianus* Blood Sample

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Bachelor of Pharmacy (Hons.)

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Declaration

It is hereby declared that

1. The thesis submitted is my own original work while completing degree at Brac University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. I have acknowledged all main sources of help.

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Approval

The thesis/project titled "HPLC in the Purification of Antimicrobial Peptides from *Andrias davidianus* Blood Sample" submitted by Abrar Kabir Khan (18146008) of Summer 2021 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of Pharmacy (Hons.) on 30/10/2022.

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Ethics Statement

No living organism were harmed during this project.

Abstract

The *Andrias davidianus* has a long history of use in traditional Chinese medicine. Its blood is regarded as a byproduct or waste of the meat industry. Although reports of the isolation of antimicrobial peptides from various sources exist, there are no records of their isolation from the blood of *A. davidianus*. In this study, Andricin B, an antimicrobial peptide, was extracted from *A. davidianus* blood using a novel technique that combines magnetic liposome adsorption with reversed-phase high-performance liquid chromatography. Further research was done on the structure, antibacterial capability, and safety of Andricin B. N-terminal sequencing was used to determine the amino acid sequence, which was discovered to be Gly-Leu-Thr-Arg-Leu-Phe-Ser-Val-Ile-Lys. A clearly defined random coil conformation was proposed by CD spectra and the three-dimensional structure prediction made by bioinformatics tools. All of the bacteria tested in this study's Andricin B tests, as well as some fungi, were shown to be resistant to it. The MICs, or minimal inhibitory concentrations, ranged from 8 to 64 g ml-1. Additionally, the haemolytic testing indicated that Andricin B might be regarded as safe at the MICs. Finally, it was discovered that Andricin B prevented *Staphylococcus aureus* from growing in cooked *A. davidianus* flesh. This work demonstrates that Andricin B is a promising new antimicrobial peptide that may offer additional insights into the creation of novel medications.

Keywords: *Andrias davidianus*; Antimicrobial peptide; Magnetic cell membrane separation; RP-HPLC

Dedication

Dedicated to all of my well wishers

Acknowledgement

First and foremost, I am thankful to Allah for allowing me to pick this subject and pursue my study on Pharmacy. Without His mighty blessings, I would be unable to complete this project to submit it in order to get my Bachelor's degree in Pharmacy.

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Table of Content

Declaration ... ii

Approval ... iii

Ethics Statement ... iv

Abstract ... v

Dedication ... vi

Acknowledgement ... vii

List of Tables ... xi

List of Figures ... xii

List of Acronyms ... xiii

Chapter 1 ... 1

Introduction ... 1

1.1 HPLC ... 1

1.2 Peptide Purification ... 1

1.3 *Andrias davidianus* ... 2

1.4 Rational of the Study ... 2

1.5 Aim of the Study ... 3

Chapter 2 ... 3

HPLC ... 3

2.1 Preparation of Mobile and Solid Phase ... 3

2.1.1 Mobile Phase Preparation 3
2.1.2 Stationary Phase Preparation 4
2.1.3 Retention in HPLC 5
2.2 Mechanism of Action 6
2.3 Types of HPLC 7
2.3.1 NP-HPLC 8
2.3.2 RP-HPLC 8
2.3.3 Ion Exchange Chromatography 10
2.3.4 Size Exclusion Chromatography 10
2.4 Advantages 11
Chapter 3 12
Peptide Purification 12
3.1 Introduction to Antimicrobial Peptides 12
3.2 Peptide Purification 13
3.3 Mechanism of Action 15
3.4 Importance 16
3.4.1 In Biologics 16
3.4.2 In Treatment 17
3.4.3 Toxicity Caused by Proteins 17
3.5 Scopes of Peptide Purification 18

Chapter 4 19

Andrias davidianus 19

4.1 Origin 19

4.2 Habitat of the Species 20

4.3 Reason behind Selection of the Species 20

4.4 Results Shown by the Antimicrobial Peptide 22

Chapter 5 23

Conclusion 23

Reference 26

List of Tables

Table 1: Commonly Used Stationary Phases and Their Associated Modes in HPLC (Sarker & Nahar, 2015) 4

List of Figures

Figure 1: Reverse-phase chromatography separation (adapted from: Salvato et al., 2012) 9

Figure 2: Conformation of Andricin B (adapted from Tang et al., 2021) 13

Figure 3: Differential induction pathways in immune response (adapted from Van ’T Hof et al., 2001) 15

Figure 4: Chinese Giant Salamander (Andrias davidianus) (left,artificially cultivated) and its fossil (right) (adapted from Zhang & Wang, 2000)... 20

Figure 5: Flow cytometry analysis of Escherichia coli CICC 10293 treated with andricin B.(adapted from Pei et al., 2019) 23

List of Acronyms

AMP antimicrobial peptide

API: Active Pharmaceutical Ingredient

BHA: Butylated Hydroxy anisole

GPC: Gel Permeation Chromatography

HPLC: High-Performance Liquid Chromatography

NP HPLC: Normal-Phase High-Performance Liquid Chromatography

PBS phosphate-buffered saline

QC: Quality Control

RP-HPLC: Reversed Phase High-performance Liquid Chromatography

SEC: Size Exclusion Chromatography

TFA trifluoroacetic acid

UV: Ultraviolet

UHPLC: Ultra High-Performance Liquid Chromatography

Variants of high-performance liquid chromatography

Chapter 1

Introduction

1.1 HPLC

High-performance liquid chromatography (HPLC) has been one of the most reliable techniques to conduct qualitative and quantitative analytical technique for about a century. The inception of chromatographic techniques can be dated back to 1903s, although back then it was as simple as paper and some solvents. But scientists soon realized the tremendous potential of chromatography. HPLC was first commercially produced by a corporation named Waters Corporation,which was dubbed as the ALC100 HPLC in 1969 (Bule, 2010). The future holds more for HPLC as we have seen progress being made by different companies in the last 20 years. Scientist are now the prime users of this company, especially in the field of bio-chemistry as the separation of proteins are rising into prominence. For the future, we want faster separation time but also more accuracy. The opportunity to attach multiple detectors to make this process even more complex and agile is multifarious. As the technology keeps on advancing, organic resins will soon play their parts in the fast and microbore columns (Bule, 2010). For only in biochemistry the scopes of HPLC are also bright in detecting pollutants, gene sequencing and to analyze the ultrafiltrates of biological molecules.

1.2 Peptide Purification

Peptides are the biomolecules that make up the proteins in our bodies. Usually, peptides are made by amino acids that form a small chain and perform specific functions in living beings. When the chain is composed of 50 amino acids, we name it proteins. These amino acids are attached to one another by peptide bonds. The term ‘peptide purification’ is often misunderstood as many take it literally. Peptide purification is a very lengthy and technical assay where at first, we must conduct

cell lysis in order to isolate the organelles from the plasma. Then they must be put in their denatured state before binding them to matrix. Finally continuous washing and eluting will ensure our desired proteins. Purification ensures that no impurities are present in our sample and also, no other type of protein should remain in the mix either. These will be explained further in details in the third chapter of the article.

1.3 *Andrias davidianus*

Andrias davidianus or as they are commonly referred to as Chinese giant salamander is the topic of our research article as the article in review discovered Andricin B, which is a peptide found to have antimicrobial properties. The research does not say that the peptide to the aforementioned species only also. The researchers have chosen this species since it is native to their land. We shall know intricate details about this reptile in the upcoming chapter.

1.4 Rational of the Study

Peptide researches are now to the utmost importance in the scientific community. The reason behind such shift of mentality arises from the future applications of biologics and biosimilar drugs. Scientist are now seeing that, in order to treat complex diseases, chemically produced synthetic drug moieties are no longer enough. So, the focus has shifted to biologics which are produced from sources of living origin or that contain molecules that closely resemble that of a bio-organism. The scientists wanted to discover a new way to identify Andricin B with the thought of reducing the cost and time to extract these peptides as efficiently as possible. Hopefully, in the future the other species under the genus of *Andrias* would be researched upon for some other peptide molecules, by the groundwork set by this article. We can also hope that, this would lead to the emergence of study in the field of biotechnology and animal life since a collaboration

between multidisciplinary fields of study is much needed to forego the advancement of science in discovering life altering and life changing molecules for a better sustainable future.

1.5 Aim of the Study

This review article was written with a view to assemble both existing ideas and foreseen strategies for peptide purification in laboratory using HPLC in order to help promote further developments. It also briefly represents the current techniques of HPLC in the field of qualitative and quantitative assessment.

Chapter 2

HPLC

2.1 Preparation of Mobile and Solid Phase

2.1.1 Mobile Phase Preparation

For this part we would be referring to the mobile phase preparation of RP-HPLC. We can prepare two different eluents by having 0.1% TFA in water and the second eluent by mixing 0.09% TFA in 60% acetonitrile/40% water (v/v) (Boysen & Hearn, 2001). We measure the organic and inorganic solvent i.e., water in two separate containers and gradually mix them otherwise errors regarding mobile phase volume may concur. Also, to get a result of our liking, we might tweak the concentration of the first eluent and the second eluent. We can change the ion pair reagent to about some percentage since organic reagents have shown to absorb more light at a lower wavelength (Boysen & Hearn, 2001). Due to the corrosive nature of TFA, we must handle it under proper supervision and use lab safety protocols, if deemed necessary. Lastly, to mix the solvents, a stirrer

of Teflon origin with magnetic flea can be used or we can gentle shake a cylinder until the mixtures are homogenous in nature.

2.1.2 Stationary Phase Preparation

While in the preparation of the stationary phase of HPLC, especially in the field of protein purification, we must ensure that,

- To run the column with a gradient run and a test mixture and
- We must maintain peptide standards for our columns for elution (Boysen & Hearn, 2001)
- We must maintain specific diameters, length and thickness of the columns.

Table 1: Commonly Used Stationary Phases and Their Associated Modes in HPLC (Sarker & Nahar, 2015)

Stationary phases	Mode
C6 silica	Reversed-phase
C8 silica	Reversed-phase
C18 silica	Reversed-phase
Silica	Normal
Diol	Normal and reversed-phase
Cyano (CN)	Normal and reversed-phase
Benzene sulphonic acid	Strong cation exchange
Polystyrene	Size exclusion

2.1.3 Retention in HPLC

Retention period is a duration of time it takes for the passage of a solute to be measured. Detection time is calculated by subtracting the time from the time of injection. Even though the similar GC and adsorbent column are utilized, a compound's RT can be affected by a variety of circumstances. The gas flow rate, oven and column temperature variations, column deterioration, and column length are only a few examples. In reversed-phase chromatography, the sample and mobile phase particles compete for adsorption on the adsorbent surface. The longer the analyte is on the surface, the more strongly it interacts with it. The chromatographic system's selectivity, or capacity to distinguish between various analytes, is also influenced by changes in the analytes' surface contacts. Today's RPLC uses dynamic surface equilibrium (DSE) as the retention mechanism. The adhesion of the sample onto the packing material is the primary equilibrium governing the retention of analytes. The analyte retention can be described in terms of this major adsorption equilibrium:

$$V_r = V_0 + SK$$

Adsorption equilibrium constant which is K, adsorbent area (S), and total column void volume (V_0) are all variables in this equation. A very small analyte concentration is assumed to have perfect sample activity in the chromatographic environment in this expression. For example, as shown in equation, equilibrium constant, K, has length units (volume/m^2) and cannot be utilized as a thermodynamic equilibrium constant (unitless). Instead, it can be used to express the retention volume per unit of adsorbent surface, such as $\mu L/m^2$. In addition to the fundamental process of analyte distribution between mobile phase and adsorbent surface, the chromatographic system undergoes numerous secondary processes that have a major impact on sample retention and selectivity. Additionally, the type of the analyte and how it appears in the mobile phase (e.g.,

ionization state) are factors that influence retention mechanisms. The sample ionization equilibrium is influenced by the eluent pH. Analyte solvation is affected by the eluent type, content, and presence of counterions. For most pharmaceutical substances, the development of separation procedures is driven by secondary processes like these equilibria, which affect analyte retention and selectivity.

2.2 Mechanism of Action

For this part of the subsection, we will only be talking about the RP-HPLC as this has been used in assessment of the peptide analysis in the research. In RP-HPLC, the stationary phase is covalently linked to alkyl or aromatic compounds to create a hydrophobic surface. A polar solvent that contains the solutes is the mobile phase that moves over the water repellent stationary phase. In this case, the basis of separation is usually formed by hydrophobic interactions between hydrophobic liquids in the mobile phase and the stationary phase. This method is called "reversed-phase" chromatography because it employs a different principle from the one that has long been employed in conventional chromatography (Dr. Catherine Shaffer, 2021).

The ability to control liquid retention and resolution by altering the mobile phase's composition is one of RP-most HPLC's potent features. In RP-HPLC, multisite reactions with the ligands are what cause peptide and protein retention. This has the practical effect of making it difficult to accomplish high resolution isocratic extraction done for peptides since the experimental range of solvent concentration needed for their elution is so small. In order to routinely elute mixtures of peptides and proteins, a gradient of additional yields of organic solvent concentration is applied. Acetonitrile, methanol, and 2-propanol are the three most often utilized organic solvents in RP-HPLC; they all exhibit great optical see-through nature in the detection wavelengths required for peptide and protein analysis. The strongest eluent is 2-propanol, and the least viscosity solvent

combinations are provided by acetonitrile. The composition of the organic solvent, in addition to its eluotropic effects, can also affect the conformation of peptides and proteins, which will have an additional impact on selectivity by modifying the conformation of the water-fearing contact zone. This may have an effect on the amount of biologically active material recovered in the case of proteins (Aguilar, 2004).

The majority of data analysis are now carried out using various PC-based programs that frequently accompany HPLC devices. To identify and/or quantify the differentiated components from a complex mixture, the retention duration, peak region, peak height, UV-Vis absorption maxima, MS data, and, in some situations, NMR data are often studied for every HPLC analysis. For quality assurance and verification purposes, data from HPLC tests can also be used for molecular fingerprinting assay of various organic product samples, including herbal items. The capabilities of HPLC software have substantially improved in recent years as a result of the enormous advancements in electronics and computational methods, making it now simple to carry out many sorts of data analysis (Sarker & Nahar, 2015).

2.3 Types of HPLC

The four primary categories of HPLC techniques are known as NP, RP, IEX, and SEC. The primary distinction between the various approaches is the focus placed on particular types of molecular interactions. In this respect, molecular forces can be classified as ionic, polar, dispersive, or repulsive. These components are used in varying proportions by each technique (Kazakevich & LoBrutto, 2006): Polar forces, a kind of intermolecular interaction, provide the backbone of the normal-phase HPLC technique.Second, in reversed-phase HPLC, dispersive forces are utilized. Ion-exchange HPLC, takes use of the fact that like ions tend to stick together.

Size-exclusion HPLC is the fourth and final type of HPLC. The stationary phase is effective because it does not react with some analytes.

2.3.1 NP-HPLC

Using this technique, the polar analytes are isolated. NP-HPLC makes use of a polar stationary phase and a non-polar mobile phase. Similar to other liquid chromatography techniques, NP HPLC separation is cost-effective. Polar stationary phase interacted with polar analyte to retain the analyte (Malviya et al., 2010). The elution times and adsorption strengths are both affected by the interactions between polar analytes and the stationary phase.

Nonpolar solvents (hexane, heptane) with a little polar modifier make up NP HPLC mobile phases (i.e., methanol, ethanol). The dose of the polar modifier in the mobile phase can be changed to alter the duration of action of the analyte in the column. Low-concentration alcohols (methanol, ethanol, or isopropanol) are an excellent illustration of a polar additive (Kazakevich & LoBrutto, 2006). For this reason, even a little shift in the mobile phase polar modifier concentration might alter analyte retention, as polar forces are the most common and strongest kind of interaction.

The solubility of a material in various mobile phases determines whether or not normal-phase HPLC should be used. Hydrophobic chemicals that are insoluble in polar or aqueous solvents are good candidates for NP because of the abundance of nonpolar solvents there.

2.3.2 RP-HPLC

Both the mobile and stationary phases are water, while the stationary phase is nonpolar. Reversed-phase HPLC relies on the repulsion between the polar extraction solvent, the non-polar analyte, and the non-polar stationary phase (RPC). Similarities exist between hydrophobic and van der Waals interactions. The quantity of analyte coupled to the stationary phase upon attachment to a

receptor in the aqueous eluent is directly related to the contact surface area around the non-polar part of the molecule.

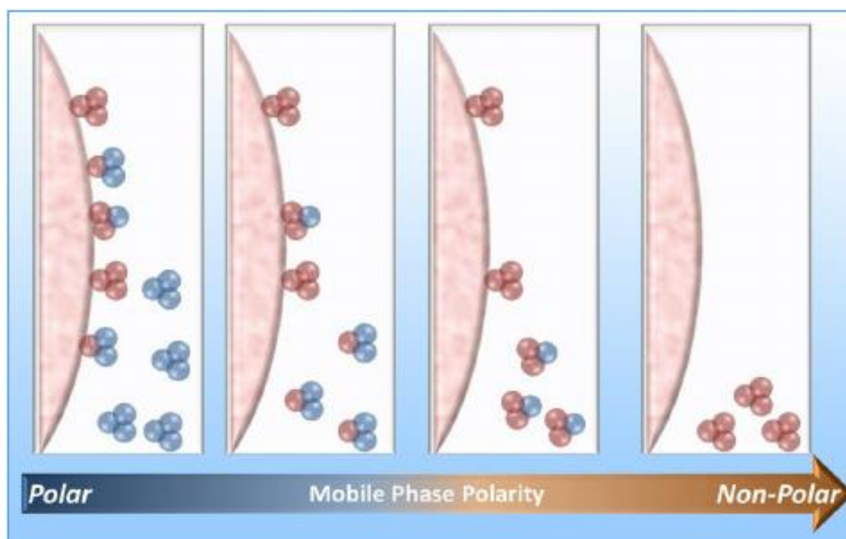

Figure 1: Reverse-phase chromatography separation (adapted from: Salvato et al., 2012)

In most cases, reversed-phase HPLC is used for chromatography. Most of the time, RP HPLC is used to examine substances with low molecular weights. Since retention and selectivity are highly flexible, it is possible to distinguish between molecules that are chemically similar. We're talking about energy savings here. Low background attractive force is typical in chromatography since the dispersive forces employed are the frailest intermolecular interactions. Molecular interactions between otherwise comparable analytes can be distinguished by their low background energy. Spectroscopy using ultraviolet light and spectroscopy using fluorescence light are quite similar. As fluorescence spectroscopy measures emission against a lower level of ambient light, it is more sensitive than ultraviolet methods. When it comes to the interaction energy between analyte and surface, RP HPLC is extremely sensitive.

Hydrophobic adsorbents are used in this chromatography technique. All HPLC techniques with significant analyte-surface contacts exhibit enhanced separation by increasing adsorbent surface

area (NP, RP, IEX). Typically, modified porous silica is used as the RP HPLC container. Over several years of research and development (Karger et al., 1967; Feibush, 1971) scientists have figured out how to make porous spheres with precisely regulated dimensions.

There has been a rise in research into chemically changing the surface of silica over the past 30 years, mostly due to the popularity of reversed phase high-performance liquid chromatography (HPLC) (Feibush, 1971; Pirkle & Hamper, 1987). For the selective analysis of pharmaceutical compounds with different ionizable functionalities, hydrophobicities, and structural components, there is still no consensus on the characteristics the ideal RP stationary phase should have despite the proliferation of packing materials and columns for RP (linear alkyl chains, aromatic rings, heterocycles, etc.)

2.3.3 Ion Exchange Chromatography

In ion-exchange chromatography, the solute ions are attracted to the charged sites on the stationary phase. Because of how pH and surface tension impact the retention and selectivity of analytes, ion-exchange chromatography is extremely sensitive. Equally charged ions are also kept out. Properties of this chromatography include water purification, ligand exchange, ion-exchange of proteins, high-pH anion-exchange of carbohydrates and oligosaccharides, and many more (Malviya et al., 2010; Dalgliesh, 1952).

2.3.4 Size Exclusion Chromatography

Gel Permeation Chromatography (GPC) and gel filtration chromatography are both names for size exclusion chromatography (SEC). It is important that the analyte not react with the stationary phase during this chromatographic separation. In size exclusion chromatography, bigger molecules elute more quickly than smaller ones because of the difference in their hydrodynamic radii. Separating molecules based on their hydrodynamic radius may be challenging if analyte molecules interact

(unwantedly) with the stationary phase, keeping bigger molecules. Both approaches have opposing consequences on a polymer's molecular weight and distribution. It's vital to choose a mobile phase and column packing material in which the mobile phase molecules interface with the stationary phase's surface more firmly than the polymer, preventing the polymer's contact. Particles of various sizes predominate. It may also be used to investigate proteins' secondary and tertiary structures. In this method, the molecular weight of polysaccharides is calculated.

2.4 Advantages

When it comes to analytical methods, HPLC is top-of-the-line. Its greatest strength lies in its adaptability. Included in this category are macromolecules, polymers, ions, and organic compounds. With HPLC and MS working together, you get the "ideal analytical instrument" in terms of separation power. Drug testing in biological ids, forensic and environmental material analysis, trace analysis of food residues, and life sciences research are just a few examples of HPLC-many MS's applications (Dong, 2006; Snyder & Kirkland, 1974; Ahuja, 2005; Kazakevich & LoBrutto, 2006). As a result of its dependability and endurance, high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection is essential for quality control (QC). Pharmaceutical research facilities frequently conduct studies on the best ways to store raw materials, intermediates, and final products (Ahuja, 2005; Kazakevich & LoBrutto, 2006). HPLC multi-segment gradients often employ ammonium formate buffer and acetonitrile as solvents. Chromatogram peaks are labeled with names like active pharmaceutical ingredient (SRR, absolute configuration for the drug molecule with three chiral centers), process impurities (SRS and RRR), degradation products (M235, M416, and M399), oxidative degradation products (ketone), and butylated hydroxyanisole (BHA) (butyl hydroxyanisole, an antioxidant additive). With three

months of storage at 50 °C/75%RH, we saw an increase in M416, SRS, RRR, ketones, and M399 degradants. As evidenced by the chromatogram (Dong, 2013).

There is nothing particularly remarkable about the chromatograms or the operating circumstances, but they do demonstrate some of HPLC's less visible properties that are relevant to quality assurance.

UHPLC has many advantages over standard HPLC, including accurate quantitation of all materials (API and similar compounds, including isomers), high reproducibility (robustness) across laboratories (utilizing equipment from multiple providers and columns from different batches), high sensitivity (ability to detect contaminants as small as 0.01% in thiols), and batch processing.

Chapter 3

Peptide Purification

3.1 Introduction to Antimicrobial Peptides

Each individual amino acid molecule in a peptide bond is known as a residue. The presence of an alpha amino group at one end and an alpha carboxyl group at the other gives a polypeptide chain its directionality. Conventionally, a polypeptide chain's amino terminal is considered the beginning of the chain, hence the amino acid sequence is always given in reverse order, with the last amino acid listed first. Therefore, tyrosine is the N-terminal (A) residue of the polypeptide Tyr-Gly-Gly-Phe-Leu, (YGGFL) while leucine is the C-terminal (C) residue. Different chemical properties are associated with the polypeptide chain Leu-Phe-Gly-Gly-Tyr (LFGGY) (Berg et al., 2015).

It has become evident over the last twenty years that in addition to bacteria and viruses, higher species also produce a wide range of antimicrobial compounds. Many of these substances are short,

cationic peptides that exhibit amphipathic activity. Because of their unique molecular architecture, they preferentially bind to phospholipid bilayers, and it is widely believed that their lethal effect is mediated by cellular membrane lysis. There is a wide diversity of antimicrobial peptides expressed by plant (Van 'T Hof et al., 2001). In this paper, Gly-Leu-Thr-Arg-Leu-Phe-Ser-Val-Ile-Lys (Pei et al., 2018) was successfully figured out from the blood sample of the amphibian species.

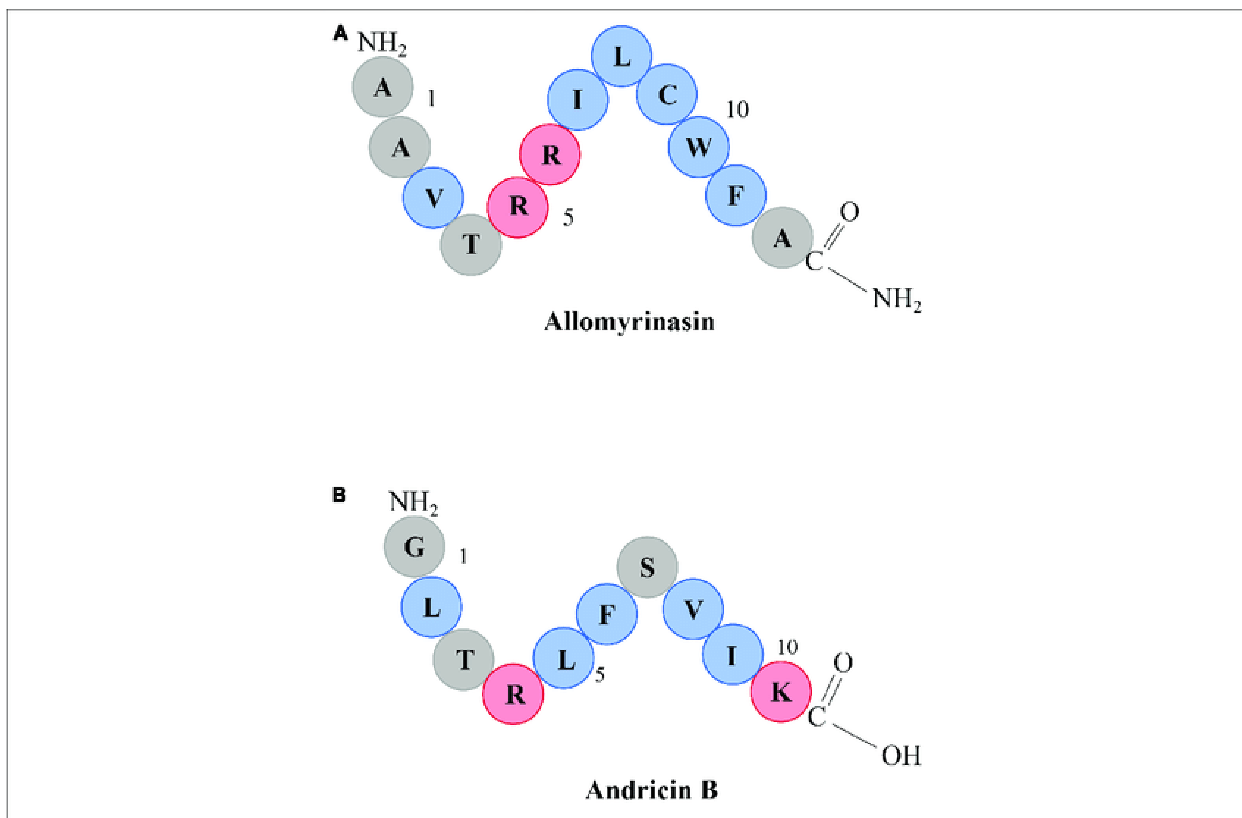

Figure 2: Conformation of Andricin B (adapted from Tang et al., 2021)

3.2 Peptide Purification

Methods applied to isolate peptide sequences are done for natural proteins, recombinant proteins produced by introducing suitable genome sequences into micro-organisms or cultivated eukaryotic

cells and lastly complex mixtures of proteins expressing due to the open field of genomics. This is more popular as proteomics, that employs large-scale isolating methods to simultaneously recognize and characterize structures are the current targets of protein purification methodologies. Purification methods routinely utilized to extract naturally produced and recombinantly produced proteins on an individual basis will be the focus of this research rather than the wide scale screening concerns addressed in the area of proteomics. (Nair et al., 1999)

Many noble techniques are being implemented now in order to elute peptides based on the many tangible quantities as these peptides are instrumental in developing and understanding the etiology of a particular set of disease. In order to sample out the must-have peptide sequence, the magnetic liposome was preconditioned with phosphate-buffered saline (PBS) (10 mmol, pH 7.2) containing 50 mmol NaCl at 37°C for 6 hours before adsorbing blood protein solutions from *Andrias davidianus*. After magnetic liposomes have been collected by magnet column, they are dissolved in a PBS (pH 7.2) buffer containing 50 mmol NaCl at 25°C to act as an isocratic mobile phase. It was found that 215 nm was the optimal wavelength for measuring absorbance. As an indicator strain, Staph. aureus CICC 10384 was used to collect and analyze the peaks for their potential bacteria killing efficacy using the spot-on-lawn method (Yue et al., 2013). The Bradford assay was used to quantify the protein content (Miao et al., 2016). The most productive apex was gathered. To get enough of the active peptides, the experiments were run multiple times. Lyophilization was then used to concentrate these active peptides. Using a gradient separation (mobile phase B: 5100%) from 0 to 5 ml min1 at 25°C, the peak with the highest activity was then screened through by RP-HPLC. Mobile phase (A) contained 0.05% (v/v) TFA, and the running phase (B) contained 100% (v/v) acetonitrile (Pei et al., 2018).

3.3 Mechanism of Action

The cationic charge and structure of AMPs are related to their microbial killing ability. AMPs are drawn to the anionic components of the lipid bilayers of bacteria, and other microbial species because of the cationic charge of the peptides. However, the interaction of the AMP with the cell receptor is only temporary, and the initial electrostatic attraction that brings the two together is not specific (Izadpanah & Gallo, 2005).

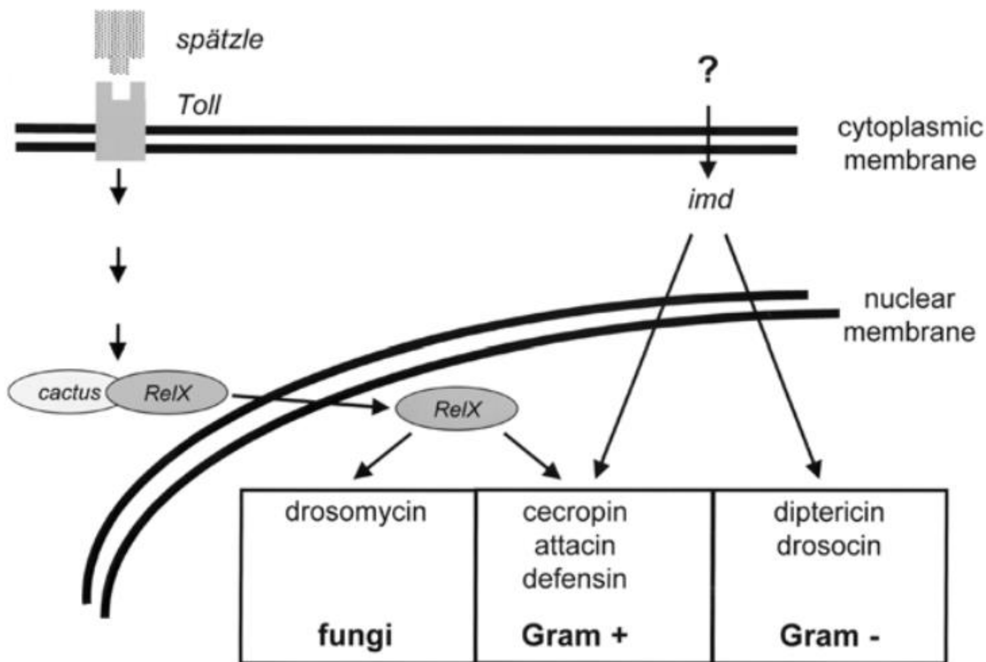

Figure 3: Differential induction pathways in immune response (adapted from Van 'T Hof et al., 2001)

Other papers state that, damage to the cell membrane is a well-known mode of action for AMPs. Electrostatic repulsion between AMPs' positively charged peptide molecule residues and the negatively charged microbial cell surfaces is one mechanism by which AMPs interact with

bacteria. It has been hypothesized that AMP specificity is determined by cell surface makeup. In this regard, the various physicochemical features of the lipids present on bacterial and eukaryotic cell membranes account for their relative sensitivities (Dathe & Wieprecht, 1999).

There is evidence that the microbicidal effects of some cationic AMPs are achieved less through direct modes of action than by the powerful induction of host immunoreactivity. Because of this, cancer immunotherapies have garnered a lot of attention in recent years due to the hope that they can be used as supplements to standard anticancer treatments (Roudi et al., 2017).

3.4 Importance

3.4.1 In Biologics

Methods involving gene therapy have been outlined, and they sound promising. Adenovirus-mediated gene transfer successfully increased LL-37 expression in a cystic fibrosis xenograft set-up, reestablishing its bactericidal function. Separate research found that human cathelicidin delivered using cutaneous adenoviral vectors was more efficient than synthetic host defense peptides in preventing infection in burn wounds. Periodontal disease, Crohn's disease, and cystic fibrosis are just few of the diseases that could benefit greatly from this new line of investigation (Guaní-Guerra et al., 2010).

In some cases, as when dealing with a specific ailment, taking extra amino acids or eating more protein in general is warranted. In contrast, a low-protein regimen may be recommended for those with kidney problems or inborn defects of the urea cycle; however, this does not necessarily equate to a reduction in protein needs. In a similar vein, a variety of inherited disorders of protein lysis can cause a deficiency in some amino acids and an increase in the need for others. Patients with phenylketonuria, for instance, need to limit their intake of dietary phenylalanine, yet tyrosine has

suddenly become crucial for them because they can't make it. Patients with inborn defects in the urea cycle also need dietary sources of arginine (save those with arginase deficiencies). Therefore, it is essential to supply proper amino acids for such and other inherited disorders of protein breakdown (Watford & Wu, 2018)

3.4.2 In Treatment

Proteins are now taking a step ahead in terms of treatment in the world. More and more mutation of each disease is leading scientist to prepare noble molecules to treat patients.Additionally, the production of epidermal cathelicidin during the progression of verruca vulgaris and condyloma accuminatum demonstrates the protective function of AMPs in skin. In mice, cutaneous multiplication of vaccinia virus corresponds with cathelicidin expression, however this has not been linked to resistance to or suppression of the transmission of human papillomavirus. A greater susceptibility to viral skin infections has been found in people with otherwise healthy immune systems, and the AMPs may shed light on this phenomenon. (Izadpanah & Gallo, 2005)

3.4.3 Toxicity Caused by Proteins

Toxic effects of protein and specific peptide chains among healthy people are poorly understood. Protein intakes up to 35% of calories tend to be accepted, but there is not enough data to determine a safe upper limit. Protein limitation may be advised in some cases. Lengthy consumption of 2 g of dietary protein per kilogram of body weight, or even a larger amount, is safe for healthy individuals, whereas children aged 1 to 3 years old can handle a dietary protein intake of 5 g per kg of body weight. Evidence of potential toxicity for some amino acids can be found in a variety of inherited metabolic disorders, but comprehensive toxicity information is scarce. At doses of 40–50 g/day, other amino acids, such as glutamine, appear to be well tolerated (Watford & Wu, 2018).

The Dietary Reference Intakes (DRI) in case of protein state, "care should be given with regard to employing any one amino acid at amounts much greater than those seen in regular meals."

3.5 Scopes of Peptide Purification

With more and more human infections developing resistance to antibiotics, there has been a push to find viable alternatives. Despite their antiquity, AMP research has only recently been prioritized as a viable therapeutic approach against harmful microbes due to their wide spectrum of activity against a diverse variety of microbial species, including bacteria and enveloped viruses. It was often considered that the bacterial membrane was the primary target of AMPs, but in recent decades, various novel and frequently combination ways of death have been identified. This type of resistance appears to be less widespread than resistance to conventional antibiotics, although it was chosen in various infections as a result of host-pathogen co-evolution. In order to construct optimal AMPS that could be effectively used as therapeutic medications, a deeper understanding of AMPs' mode of action and opposing resistance mechanisms is essential (Guilhelmelli et al., 2013).

Using human cathelicidin LL-37, tachyplesin 1, and the synthetic AMP D2A21 as chemical dAMP standards, RP557 was the result of three iterative functionalized cycles of peptide synthesis. Two disulfide connections are crucial to the improved activity and stability of RP557, and they also serve to lock the protein into its active shape. When incubated for 72 hours at 37 degrees Celsius, RP557 showed no signs of degradation, demonstrating its resistance to proteolysis and hence highlighting its improved stability. More importantly, three freeze-thaw cycles showed no deterioration in RP577's quality (Woodburn et al., 2019).

Researchers interested in AMP-activated protein kinase now include specialists in oncology, diabetes, cardiomyopathy, and overweight. The need for powerful, selective AMP-activated protein kinase medicines that can be given to patients or utilized as tool molecules for in vitro investigations has been a recurrent and unifying topic in the AMP-activated protein kinase literature.

Chapter 4

Andrias davidianus

4.1 Origin

Chinese Giant Salamander, as the name suggests is local to the country of China and its roots can be traced back to the Jurassic period and it is reported that the family of cryptobranchidae got isolated from other amphibians during that timeline (Chinese Giant Salamander - EDGE of Existence, 2013). The taxonomy of the Chinese salamander is that it belongs to the class of amphibia as they are both terrestrial and also live in water bodies, their order is caudata as their characteristic body feature is the long tail that consists almost 60% of the body's total length, their family is cryptobranchidae as they have costal grooves which is important to diffuse water into their body thus keeping their skin cool and damp and their genus is andrias as they are one of the largest amphibians in the world (Chinese Giant Salamander - EDGE of Existence, 2013), the species of *Andrias davidianus* being the largest amphibian in the world. The Chinese salamander has some other species which closely resemble our selected species which are the Japanese Giant salamander and lastly American Hellbender. It's a matter of great sorrow because these three are

the only remaining living species of salamander which are of such stature in cryptobranchidae family as they are constantly hunted for their meat and their value in the market of pharmaceuticals.

Figure 4: Chinese Giant Salamander (Andrias davidianus) (left,artificially cultivated) and its fossil (right) (adapted from Zhang & Wang, 2000)

4.2 Habitat of the Species

For the Chinese Giant Salamander, a huge hill stream is where it dwells and lays its eggs. When the female lays around 500 eggs in a row in an underwater chamber, the male takes up residence. The eggs are externally fertilized by a patrolling male and hatch in 50-60 days (Sparreboom, 2000). After around 30 days, the larvae are ready to eat. During the mating season, the species may be seen during the daytime, despite its usual nocturnal habits. Small animals and reptiles are also prey for these fish-eating mollusks and crustaceans.

4.3 Reason behind Selection of the Species

Chinese Giant Salamander (CGS) are a very mythical creature to the Chinese region and the Chinese have discovered a lot of benefits of consuming the meat of these great amphibians. These species have been long documented as a type of 'fish' in ancient scriptures of Chinese herbal

medicinal books and people used to be believe that the meat could help with dementia and dysentery. Consumption of CGS meat was also done to boost Qi which is spiritual energy, in the beliefs of the Chinese. But modern scientists have seen that the meat of CGS contain properties like anti-oxidant, anti- aging, anti-tumor, anti-fatigue and so on (He et al., 2018). In order to counteract the detrimental attributes of free radicals and many oxidizing agents on the human body, it is essential that we eat foods rich in antioxidants. Many investigations have shown that protease breakdown of CGS muscle and mucus can provide active polypeptides with considerable antioxidant activity (He et al., 2018). Mucus polypeptide molecular weight, reproductive age, and enzymolysis conditions all influence CGS polypeptide antioxidant activity. In the field of anti-tumor, which is an alarming threat in the modern world and mostly related to the development of toxins and pollutants that we either inhale or consume in the form of adulterated food particles and that causes buildup of free radicals in the cells resulting in cancer cell formation. Extracts of CGS have been proven to reduce the functions of lipofuscin thus helping to slow down the process of anti-aging without damaging the reproductive health of test subjects (He et al., 2018). In regards of the anti-fatigue properties, experiments demonstrated that the test subjects could swim for longer, with lower lactate levels, and with more hepatic glycogen when given CGS oligosaccharide peptide. This indicates the substantial anti-fatigue impact that CGS has on the test subjects. Also, in the terms of the anti-microbial effects, an experiment was conducted where three ointments, "nisugao," "niyougao," and "nipigao," were made from the mucus, fat, and dermis of CGS, sesame oil, and beeswax. Second-degree burn and cold-blisters wound healing times were evaluated using the experimental mouse model and the scientist also chose a control group in the form of Mebo burn ointment, which is a commercially available product (He et al., 2018). Mebo's burn ointment

was shown to be ineffective in promoting wound healing in both scars and frostbite enticed mice, according to the results of the experiment.

4.4 Results Shown by the Antimicrobial Peptide

Antibacterial peptides often have limited pH stability (Li et al. 2012; Yue et al. 2013; Martinez et al. 2015). This inactivity is not observed with Andricin B, according to our spectrum data. Despite the fact that the Andricin B structural alterations triggered by sensitive bacteria vary depending on the pH of the surrounding environment. Commonly, one inflection is heard (between 220 and 240 nm). This finding raises the possibility that the inflection is crucial to Andricin B's antibacterial action. At physiological pH, both the Arg and Lys sites in Andricin B become protonated. Due to their shared negative charge, this peptide should bind tightly to their bacterial counterparts. Evidence from the past suggests that when exposed to sensitive bacteria, some antimicrobial peptides adopt rigid and particular conformations (Paiva et al. 2012; Pu and Tang 2016; Pei et al. 2018b).

The fluorescence intensity of FDA dropped as Andricin B was added to E. coli cells, but that of PI rose. Based on these findings, it appears that lactonases in cells are being inactivated and membranes are being destroyed. But there was no coordination between these processes. Cellular lactonases were rendered inactive after 1 hour. Furthermore, there had been no appreciable upsurge in PI fluorescence (Pei et al., 2019). Even after 4 hours of incubation, the PI concentration in the cells was still rising. Sublethal damage was identified by using PI/FDA double-stained cells (FDA+ PI+). Sublethal cells also grew steadily as incubation times lengthened. These findings suggested that bacterial cells exposed to Andricin B underwent a sublethal phase before dying.

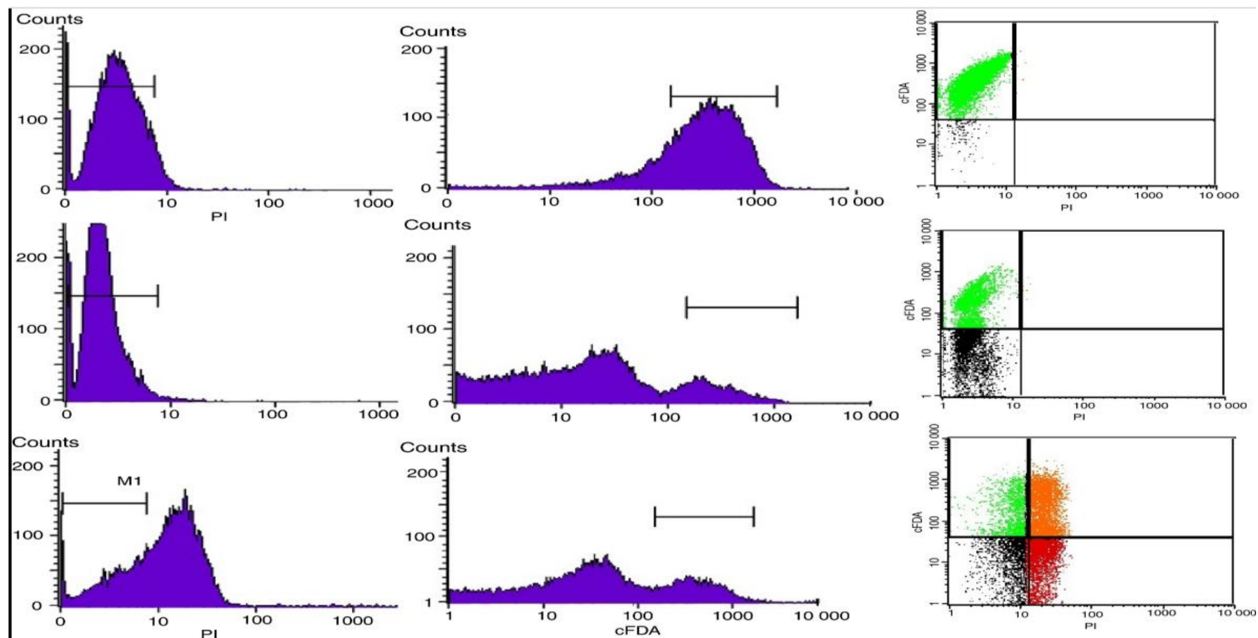

Figure 5: Flow cytometry analysis of Escherichia coli CICC 10293 treated with Andricin B.(adapted from Pei et al., 2019)

Data showed that the molecular foci on Andricin B-sensitive bacteria were located in the cell wall sugar, the mixed areas, and the protein amide regions. Andricin B appears to cause alterations in the membrane characteristics of certain parts of the cells (Pei et al., 2019).

Chapter 5

Conclusion

AMP therapy has the potential to be a promising new method of treatment. Many animal studies have demonstrated the importance of cell treatment, and because of their cell-to-cell specific potential, ability to undergo in vivo, and cell membrane lytic mechanisms of action, AMPs have been regarded as a prominent way to combat diseases. Use of AMP for bactericidal effects is by

far, the most recent options, with promising safety profiles in human trials, despite the relative fact that due to their small size, they can possibly bind to other receptor sites. the collection method of AMPs is also very complex and sophisticated have the benefits of being relatively simple to collect and culture, being effectively low-cost and customized treatment options for patients with renal damage. Furthermore, health concerns from AMP treatment have been documented in animal experiments, and the majority of evidence indicates that cells engraftment into damaged organs is temporary. The injection of AMPs has not been associated to any severe side effects in clinical trials. Besides that, to establish the long-term safety of these therapies and to assess the hazards of fibrosis, mal-differentiation, or cancer, carefully designed experimental trials are required. Only larger, well-powered, properly conducted clinical trials will be able to assess the true therapeutic efficacy of AMP treatment in illness. Technological challenges and high costs of production have discouraged pharmaceutical companies from putting significant resources into the creation of antibiotic peptide therapeutics. However, a variety of antibiotics have discovered peptides having medicinal potential within the past twenty years. The one-of-a-kind pharmacological inherent characteristics has restricted its usage to the skin in exceptions where no non-peptide treatment option exists, or where the negative effects of using standard treatment are too great to be tolerated. These medications are high-priced specialty pharmaceuticals having either a narrow therapeutic index or inadequate safety data for widespread usage, sufficiently large to be of commercial interest. The greatest difficulty o eventual goal of pharmaceutical to overcome the potential restrictions of these intriguing compounds and to create them into medicines with multiple applications of uses, and at a fair price. AMPs are appealing as therapeutic tools because they not only possess antibacterial activity, but also a wide variety of other physiologic actions that play a role in the regulation of infectious and allergic illnesses. It is possible to use AMPs in humans

through their synthesis or the creation of analogues. Inducing the endogenous creation of these peptides is an intriguing alternative since it would circumvent potential toxicity and undesirable systemic reactions and the challenge of delivering them in integral form to the intended locations of action. Diseases like cystic fibrosis and Crohn's can be treated with AMPs because of their ability to replace or restore the function of dysfunctional endogenous peptides. However, high or persistent levels of these peptides may contribute to a chronic inflammatory process, as seen for psoriasis and rosacea, therefore careful management of them should be addressed. But one of the biggest problems in contemporary medicine is the spread of microorganisms that are resistant to antibiotics. There is undeniable requirement for innovative therapies that are both effective and safe. Since AMPs have been found to be effective against bacterial, viral, and fungal infections, they may one day serve as important therapeutic tools. In addition, their multifunctional qualities allow their antimicrobial activity to be exerted in a variety of ways, which makes resistance development in microbes more challenging.

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