



EXTRACTION OF PROTEIN CONTENT FROM COLLAGEN

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ABSTRACT

Collagen, a vital structural protein found abundantly in connective tissues, has garnered significant attention in biomedical research due to its diverse applications in tissue engineering, regenerative medicine, and drug delivery systems. Traditional methods of collagen extraction often involve harsh chemical treatments, leading to protein denaturation and reduced biological activity. This thesis explores innovative approaches to collagen protein extraction, focusing on techniques that preserve structural integrity and enhance bioactivity. The literature review highlights recent advancements in collagen extraction methods, including enzymatic hydrolysis and supercritical fluid extraction, which offer superior yield, purity, and retention of bioactivity compared to conventional methods. Methodological approaches for each extraction technique are detailed, outlining specific parameters and analytical methods for evaluating collagen quality. Experimental results demonstrate the efficacy of novel extraction methods in producing high-quality collagen with enhanced bioactivity, as confirmed by SDS-PAGE analysis, FTIR spectroscopy, and cell culture assays. Comparative analysis reveals the advantages of enzymatic hydrolysis and supercritical fluid extraction over traditional chemical methods, Creating a foundation for their broad acceptance and use in biomedical research and clinical applications. The findings of this thesis contribute to the advancement of collagen extraction technologies, providing researchers and provide clinicians with important insights into optimizing collagen production for various biomedical applications. Potential areas for future research involve investigating novel biomaterial formulations and scaffolding techniques to harness the full therapeutic potential of collagen in tissue engineering and regenerative medicine. This thesis explores recent advancements in collagen extraction techniques, with a focus on strategies aimed at optimizing yield, purity, and functional properties for biomedical applications. A comprehensive review of the literature elucidates the evolution of collagen extraction methodologies, highlighting the strengths and the constraints of conventional approaches and the emergence of innovative methods that show potential for overcoming existing barriers. Enzymatic hydrolysis, a non-destructive method employing specific proteases to cleave peptide bonds within collagen molecules, has become popular due to its capability to produce high-quality collagen with minimal degradation and superior bioactivity. Supercritical fluid extraction (SFE), utilizing CO₂ as a solvent under controlled pressure and temperature conditions, offers a solvent-free and environmentally friendly alternative to conventional extraction methods, resulting in collagen products of exceptional purity and stability. Methodological insights and experimental findings from comparative studies underscore the advantages of enzymatic hydrolysis and SFE over traditional chemical methods, demonstrating their efficacy in preserving the structural integrity and biological functionality of collagen. Analytical techniques such as SDS-PAGE, FTIR spectroscopy, and cell-based assays provide valuable insights into the composition, conformation, and bioactivity of extracted collagen, enabling researchers to optimize extraction protocols and customize collagen-based biomaterials for specific biomedical application.

Introduction

A fibrous structural protein is collagen. that is the core element of connective tissues in the bodies of animals, including humans. It is The most prevalent type of protein in mammals, making up a significant portion of the skin, bones, tendons, ligaments, and other connective tissues.

Beyond its role in maintaining tissue integrity, collagen is essential for skin elasticity, joint flexibility, and overall organ function. Understanding collagen is crucial for advancements in fields like medicine, cosmetics, and biomaterials.

Collagen is a fibrous protein abundantly found in connective tissues, such as skin, tendons, and bones. Its importance lies in providing structural support and strength to various body parts.

Among them is collagen crucial protein that acts as a resource primary building block in the connective tissues of animals, including humans. It is characterized by its fibrous structure and plays a fundamental role in providing strength, structure, and support to various tissues and organ systems inside the organism. The key features of collagen include its prevalence in skin, bones, tendons, ligaments, and cartilage, as well as its diverse types (e.g., Type I, Type II, Type III) They are tailored for various functions.

The synthesis of collagen involves a complex process within cells, resulting in the formation of triple-helix chains of amino acids. Collagen undergoes continuous turnover, where it is broken down and replaced, contributing to tissue health and adaptability. The protein has garnered widespread interest in fields such as medicine, cosmetics, and research because of its applications in tissue engineering, wound healing, and anti-aging products.

Understanding collagen is essential for advancements in various industries, and its significance extends to areas like skincare, medical implants, and dietary supplements. Overall, collagen's versatile and foundational role in the body makes it a subject of substantial scientific and practical interest.

Collagen, a fibrous protein present in all multicellular animals, serves as a crucial structural component in both vertebrates and invertebrates. It constitutes the majority of proteins in mammals, accounting for roughly 25% of their total protein weight, and plays a vital role in various tissues including skin, tendons, cartilage, and bones. In poultry and fish, collagen fulfills similar structural functions as invertebrates, being a key element of the body wall.

The Collagen molecular types are approximately 280 nm in length with a molecular weight of 360,000 Da. Hydrogen stabilizes them. and intermolecular bonds, comprised including three polypeptide chains with helices, each having roughly 1000 amino acids known as α chains. These chains intertwine in order to create stable triple helix structure called procollagen, which is further processed into tropocollagen, the fundamental unit of collagen, through cleavage of globular domains. Hydrophobic and electrostatic interactions stabilize the molecules of tropocollagen. interactions.

Vertebrates exhibit various types of collagen, typically rich in glycine, alanine, proline, and hydroxyproline. The amino acid sequence in collagen follows a repetitive tripeptide pattern (Gly-X-Y), with X often being proline and Y being hydroxyproline. Over 29 Several varieties of collagen have been identified, categorized based on their structure into fibrous, network forming, filamentous, and those associated with fibrils.

Type I collagen is the most prevalent, found predominantly in connective tissues such as skin, tendons, and bones. Type II collagen is primarily present in cartilage tissue. Type III collagen degrees change dramatically with age., being abundant in very young skin but decreasing as individuals age. There are additional forms of collagen in smaller quantities, primarily in specific organs like Muscle of the heart, cornea, and basement membranes, lungs, and gastrointestinal mucosa.

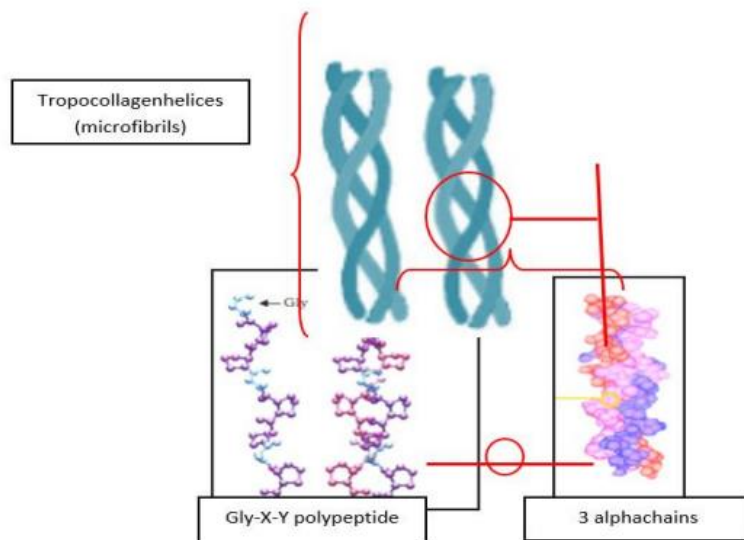


Figure 1.1 - The composition of Connective tissue

Among them is collagen, a triple-standard helical molecule that is an important part of the connective tissue and extracellular matrix tissues in animals. **Bruckner, P. (1987).**

Key Features of Collagen.

Fibrous Structure: Collagen is characterized by its fibrous, rope-like structure. It forms long, triple-helix chains of amino acids, creating a strong and flexible framework.

Function: The primary function of collagen is to provide strength, structure, and support to tissues and organs. It plays a crucial role in maintaining the health of blood vessels, bones, cartilage, and skin, and other connective tissues.

Types: Various kinds of collagen, each with certain positions in the body. For example, Type I collagen is predominant in skin, bones, and tendons, while Type II is found in cartilage, and Type III is present in reticular fibres.

Synthesis: Collagen is synthesized within cells called fibroblasts. The synthesis involves a complex process of assembling individual amino acids into long chains, which then form the characteristic triple-helix structure.

Degradation and Renewal: Collagen is constantly being broken down and replaced in the body through a process called remodelling. This turnover is crucial for maintaining tissue health and adaptability.

Understanding collagen is critical in various scientific and medical fields, contributing to advancements in regenerative medicine, biomaterials, and therapies for conditions related to connective tissues.

Types Of Collagens

Type I Collagen

Location: Skin, tendons, bones, Ligaments, and the organic part of teeth.

Function: Provides tensile strength and has the greatest abundance. type of collagen in the human body.

Type II Collagen

Location: Cartilage, the vitreous humour of the eye, and the intervertebral discs.

Function: Offers structural support, especially in cartilage tissues.

Type III Collagen

Location: Skin, blood vessels, and internal organs.

Function: Often found in association with Type I collagen, providing structural support.

Type IV Collagen

Location: Basement membranes, which underlie epithelial and endothelial cells.

Function: Forms a mesh-like structure, contributing to the structural integrity of basement membranes.

Component of Collagen

Collagen is a complex protein with a unique structure, and its primary components are amino acids. The specific amino acid composition gives collagen its distinctive properties.

Amino Acids: Collagen is composed of amino acids, which constitute the fundamental components of proteins. The most abundant Glycine, proline, and hydroxyproline are the amino acids found in collagen, and arginine. Glycine is particularly crucial, accounting for about one-third collagen's amino acid composition.

Triple Helix Structure: The unique and defining feature of collagen is its triple helix structure. This structure is formed by three polypeptide chains, or alpha chains, intertwined around each other in a helical fashion. The repeating amino acid sequence, often characterized by the motif X-Y Gly (in which X and Y are often proline and hydroxyproline), contributes to the stability of the helix.

Hydroxyproline: Hydroxyproline is a modified form of the amino acid proline. It plays a crucial role in stabilizing the collagen structure by forming hydrogen bonds within and between collagen molecules. Hydroxyproline is essential for the proper function and integrity of collagen.

Collagen Fibrils: Collagen molecules assemble into larger structures called fibrils. Fibrils are formed by the alignment and bundling of collagen molecules, providing strength and support to tissues.

Collagen Types: There are various types of collagen, every one having a distinct distribution and makeup. Where X and Y are in Gly-X-Y in the body. For example, Type I collagen is abundant in skin, bones, and tendons, while Type II is found in cartilage. The types of collagen are classified based on their distinct alpha chain compositions.

Cross-Linking: Collagen molecules are often cross-linked, establishment of covalent connections between nearby chains. This cross-linking enhances the strength and stability of collagen fibrils and contributes to the resilience of connective tissues.

Non-Collagenous Protein: In addition to collagen, connective tissues may contain non-collagenous proteins that interact with collagen and contribute to the overall properties of the tissue. These proteins may include proteoglycans, glycoproteins, and other matrix proteins.

Minerals: In certain tissues, such as bone, collagen may also contain mineral components, such as hydroxyapatite crystals. This mineralization process adds further strength and rigidity to the tissue.

Collagen Isolation and Characterization

Various kinds of collagen may be taken out of several sources utilizing methods such as neutral salt extraction, acid extraction, and enzymatic extraction. These methods involve suspending the source materials in cold water, chopping them up into little bits, and then subjecting them to different extraction solutions. For instance, neutral salt extraction involves gradually increasing salt concentration, while acid extraction uses organic acids. Enzymatic extraction involves combining organic acids with pepsin. Despite variations in extraction efficiency, acid extraction generally yields higher amounts of collagen compared to neutral salt extraction.

Collagen composition varies depending on the extraction method and the source material. Proline, glycine, and hydroxyproline are abundant in collagen, forming characteristic triple-helical repeats. Additionally, alanine and hydroxyproline levels are notable in fish scale collagens. However, the degree of hydroxylation of proline can vary, affecting collagen fibre stability and denaturation temperatures.

The thermal properties of collagen, including denaturation temperature (DT), are influenced by factors like amino acid content and environmental conditions. Collagen from cold-water fish typically has lower amino acid content and thus lower stability. Acetic acid treatment can further alter collagen's thermal properties by disrupting hydrogen bonds, leading to decreased stability.

Protein

Proteins, fundamental macromolecules in living organisms, orchestrate a myriad of biological processes, functioning as the molecular machinery of life. Composed of amino acids, proteins exhibit remarkable structural diversity and dynamic functionality, enabling them to serve as catalysts, structural components, transporters, and signalling molecules.

This introduction delves into the fundamental characteristics, structures, and functions that define proteins in the intricate tapestry of cellular life.

Structural Diversity of Amino Acids: Proteins are constructed from a set of 20 amino acids, each characterized by a unique side chain. The sequence and arrangement of these amino acids dictate the three-dimensional structure and, consequently, the function of the protein.

Primary Structure: The linear sequence of amino acids constitutes the primary structure of a protein. This sequence is genetically encoded and serves as the foundation for the subsequent levels of structural organization.

Secondary Structure: A protein's secondary structure emerges through local folding patterns, mainly alpha helices and beta sheets. These structural motifs originate from the interactions between hydrogen bonds in amino acid residues.

Tertiary Structure: The overall three-dimensional shape of a protein, known as its tertiary structure, arises from intricate folding and relationships between the side chains of amino acids. Numerous factors, such as hydrogen bonding, hydrophobic interactions, and disulfide bridges, contribute to this level of organization.

Quaternary Structure: Some proteins, known as multimeric or oligomeric proteins, exhibit quaternary structure. This involves the arrangement and interaction of multiple protein subunits to form a functional, larger protein complex.

Functional Diversity: Proteins serve an array of biological functions. Enzymes catalyze biochemical reactions, structural proteins provide support to cells and tissues, transport proteins move molecules within the body, and signaling proteins regulate cellular communication.

Dynamic Nature: Proteins are dynamic entities, capable of undergoing conformational changes in response to environmental cues or interaction with other molecules. This inherent flexibility is vital for their diverse functions.

Genetic Information and Protein Synthesis: The genetic information encoded in DNA is transcribed into messenger RNA (mRNA), which, in turn, directs the synthesis of proteins through a process called translation. Ribosomes, the cellular machinery, facilitate this intricate dance of molecular synthesis.

Diseases and Therapeutic Targets: Dysregulation or mutations in proteins can lead to various diseases. Understanding protein structures and functions is crucial in drug development, as many pharmaceuticals target specific proteins to modulate cellular processes and treat ailments.

In essence, proteins stand as the cornerstone of biological complexity, bridging the realms of structure and function to sustain life at the molecular level. This exploration into the world of proteins forms the foundation for unravelling the intricacies of cellular mechanisms and the broader landscape of biological systems.

Protein Synthesis: A Molecular Symphony:

Protein synthesis, a fundamental cellular process, is a complex and highly regulated mechanism that orchestrates the creation of proteins, the molecular architects of life. This intricate dance of molecular interactions involves transcription of genetic information into messenger RNA (mRNA) and its subsequent translation into functional proteins. This overview unravels the stages of protein synthesis, from the transcription of DNA to the final assembly of the protein product.

Transcription: Blueprint from DNA to mRNA: A combination of proteins begins with transcription, a process occurring in the confinement nucleus. Here, the DNA unwinds, also, an enzyme known as RNA polymerase interprets the DNA template strand, synthesizing a complementary mRNA strand. This newly formed mRNA, carrying the genetic code, acts as a transient copy of the gene.

mRNA process: Refining the Script: The initial mRNA transcript undergoes processing steps to refine its structure. Introns (non-coding regions) are excised, and exons (coding regions) are spliced together, forming a mature mRNA molecule ready for translation. A protective cap is added at the 5' end, and a poly-A tail at the 3' end enhances mRNA stability.

Translation: Decoding the mRNA message: Translation, the second phase of protein synthesis, takes place in the cytoplasm at cellular structures called ribosomes. Transfer RNA (tRNA) molecules ferry proteins to the ribosome, aligning them in light of the mRNA codons. The ribosome catalyzes the formation forming peptide connections between neighboring amino acids, elongating the growing polypeptide chain.

Initiation, Elongation and Termination: The Ribosomal Ballet: Translation unfolds in three main stages. Initiation involves the assembly of the ribosome on the mRNA start codon. Elongation sees the sequential addition of amino acids to the growing polypeptide chain. Termination occurs when a stop codon is reached, signalling the release of the completed polypeptide.

Protein Folding and Modification: Sculpting the Final Form: Once synthesized, the polypeptide chain undergoes intricate folding to adopt its functional three-dimensional structure. Chaperone proteins assist in this process, ensuring proper folding and preventing misfolding. Post-translational modifications, such as phosphorylation or glycosylation, may further refine protein structure and function.

Quality Control: Guardians of Cellular Integrity: Cells employ quality control mechanisms in order to track and rectify errors in protein synthesis. Misfolded or aberrant proteins are targeted for degradation by cellular machinery, maintaining the integrity of cellular function.

Protein Targeting and Localization: Navigating Cellular Territories: Proteins are directed to specific cellular compartments based on signals within their amino acid sequences. This ensures that proteins reach their designated locations, whether within the cell, on the cell surface, or in extracellular spaces.

Diverse Functions of Proteins: The Cellular Workforce: The synthesized proteins play diverse roles in cellular functions, serving as enzymes catalysing biochemical reactions, structural elements providing support, transporters facilitating molecular movement, and signaling molecules orchestrating cellular communication.

Protein synthesis, an intricate molecular ballet, lies at the heart of cellular processes, shaping the diversity and functionality of life. This finely tuned symphony of genetic information, transcription, and translation underscores the complexity of cellular life and the remarkable abilities of cells to create and regulate the molecular machinery essential for their survival.

Protein extraction from collagen is a crucial process in various fields, including biomedical research, regenerative medicine, and the production of biomaterials. Collagen, a fibrous protein abundant in connective tissues, serves as a structural framework in the body. Extracting proteins from collagen involves breaking down the collagen matrix to isolate and obtain specific protein fractions.

The choice of collagen source is essential and depends on the intended application. Collagen usually taken out of animal tissues such as skin, tendons, bones, or fish scales. Different sources may yield collagen with distinct properties.

Before extraction, the collagen source undergoes pre-treatment to remove non-collagenous components such as fats, cells, and minerals. This step is crucial for obtaining pure collagen.

Enzymatic methods involve the use of proteases such as pepsin and trypsin, or collagenase to break down collagen into its constituent proteins. Enzymatic extraction is known for its specificity, targeting collagen without affecting other proteins.

Chemical methods typically involve the use of acids or bases to solubilize collagen. Acidic solutions, such as acetic acid or hydrochloric acid, can be employed for collagen extraction. Alkaline solutions, like sodium hydroxide, are also used. Chemical extraction is versatile but may be less selective than enzymatic methods.

In some cases, mechanical methods such as grinding or homogenization may be employed to disrupt the collagen matrix and facilitate protein extraction.

The extracted protein solution undergoes filtration and centrifugation to remove insoluble debris and obtain a clarified collagen protein solution.

Dialysis is often employed to remove residual salts and other small molecules from the extracted protein solution, resulting in a purified collagen protein sample.

The extracted collagen proteins are characterized using techniques such as sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), which separates proteins based on their molecular weight. Other analyses, including Fourier-transform infrared spectroscopy (FTIR) or circular dichroism, can be used to assess protein structure.

The final extracted collagen proteins are typically stored at low temperatures to maintain stability until further use.

The extracted collagen proteins find diverse applications, including the progression of biomaterials, scaffolds for tissue engineering, convalescence materials, and cosmetic products.

Challenges in protein extraction from collagen include the risk of denaturation, variability in collagen sources, and the possibility of immunogenic responses in certain applications.

Recent innovations include the progress of synthetic collagens, computational modeling to predict denaturation risks, and the exploration of eco-friendly extraction methods.

The Importance of Protein Extraction: Unlocking the Molecular Treasures

Protein extraction is a pivotal scientific process with profound implications throughout a range of sectors, from medicine and biotechnology to nutrition and beyond. This section elucidates the multifaceted importance of protein extraction, shedding light on its crucial role in unravelling the mysteries of cellular life and harnessing the potential of proteins for various applications.

Advancing Biomedical Research:

In the realm of biomedical research, protein extraction stands as a gateway to understanding the intricacies of cellular function. Proteins, as the molecular architects of life, orchestrate cellular processes, and extracting them unveils a trove of information. Researchers delve into the realms of proteomics, dissecting the protein landscape to decipher disease mechanisms, identify biomarkers, and formulate targeted therapeutic interventions.

Biomaterials and Tissue Engineering:

Proteins extracted from collagen, a paramount structural protein, serve as building blocks for innovative biomaterials. From scaffolds for tissue engineering to matrices for regenerative medicine, protein extraction from collagen-rich sources enables the creation of materials that mimic the natural environment, fostering cellular growth and tissue regeneration.

Cosmetic and Dermatology:

The cosmetic industry harnesses the benefits of protein extraction for the formulation of skincare products. Collagen-derived proteins contribute to anti-aging formulations, promoting skin elasticity and hydration. Extraction techniques play a pivotal role in preserving the bioactivity of these proteins, ensuring their efficacy in skincare applications.

Nutritional Sciences:

Protein extraction extends its reach into nutritional sciences, where proteins that come from different resources become essential components of dietary supplements and functional foods. Understanding extraction methods is paramount in preserving the nutritional value and bioavailability of proteins, addressing global challenges related to malnutrition and dietary deficiencies.

Pharmacology and Drug Development:

In the pursuit of novel therapeutic agents, protein extraction becomes a linchpin in drug development. Isolating and characterizing proteins from natural sources or engineered systems provides researchers with the raw materials for formulating pharmaceuticals. The structural and functional insights gained from protein extraction guide the design of targeted drugs with enhanced efficacy and reduced side effects.

Environmental and Industrial Applications:

Beyond the realms of biology and medicine, protein extraction finds application in environmental sciences and industrial processes. Enzymes, often extracted proteins, catalyze reactions in diverse fields, from wastewater treatment to biofuel production. Protein extraction methodologies contribute to optimizing enzyme yields and activity.

Innovation in Agriculture:

Proteins play pivotal roles in plant and animal systems, influencing growth, development, and resistance to environmental stress. Protein extraction techniques contribute to understanding these roles, facilitating innovations in agriculture, such as the development of genetically modified crops or the enhancement of livestock for improved yields.

Global Impact and Sustainability:

As the global demand for proteins increases, sustainable and efficient extraction methods become imperative. Protein extraction research intersects with the broader goals of sustainable agriculture, responsible resource utilization, and eco-friendly production practices, contributing to the global pursuit of a more sustainable and resilient future.

Unlocking the Potential: The Crucial Role of protein Extraction from Collagen

Protein extraction from collagen stands at the forefront of scientific exploration, offering a key to unlocking the versatile applications of these molecular treasures. As collagen, a fundamental protein in the extracellular matrix, weaves its intricate web throughout various tissues, the extraction of proteins from this matrix becomes a pivotal endeavour with far-reaching implications across medicine, cosmetics, and biomaterials.

Relevance in Medicine

Proteins Extracted from collagen hold immense significance in the realm of medicine. Collagen-derived proteins are integral to understanding cellular processes, disease mechanisms, and the identification of potential therapeutic targets. Researchers delve into the proteomic landscape, unravelling the complex interplay of proteins to decode diseases at the molecular level. The extracted proteins become crucial building blocks for the development of targeted therapies, personalized medicine, and advancements in regenerative medicine. From biomarker discovery to innovative drug formulations, the extracted proteins from collagen contribute to the forefront of biomedical research, paving the way for transformative breakthroughs.

Cosmetic Innovations

In the realm of cosmetics, protein extraction from collagen is a cornerstone for formulating products that transcend traditional skincare. Collagen-derived proteins, rich in bioactive compounds, hold a crucial position in promoting skin elasticity, hydration, and overall rejuvenation. Protein extraction techniques ensure the preservation of these bioactive properties, providing the cosmetic industry with the raw materials for crafting anti-aging formulations. From serums to creams, collagen-derived proteins become essential ingredients, transforming the landscape of skincare and offering consumers innovative solutions for maintaining skin health and vitality.

Biomaterials Innovation

The applications of extracted proteins extend into field of biomaterials, where collagen-derived proteins become the scaffolding for innovation. Protein extraction lays the foundation for creating biomaterials that mimic the natural extracellular matrix, fostering cellular growth and tissue regeneration. From tissue engineering to regenerative medicine, the extracted proteins provide the structural framework for building artificial tissues, implants, and matrices. Understanding the intricacies of protein extraction ensures the bioactivity and biocompatibility of these biomaterials, driving progress in the creation of implants, wound healing technologies, and innovative solutions for tissue repair.

In essence, the extraction of proteins from collagen transcends the laboratory bench, influencing the forefronts of medicine, cosmetics, and biomaterials. The relevance of these extractions lies not only in unravelling the mysteries of cellular life but also in harnessing the potential for tangible applications that impact human health and well-being. As researchers refine extraction techniques and delve deeper into the functional properties of collagen-derived proteins, the journey of exploration continues, promising a future where the applications of these extracted proteins will reshape the landscapes of medicine, cosmetics, and biomaterials.

Research Gap and Motivation

Bridging Gaps, lighting Motivation: A Journey into Protein Extraction from Collagen

Gaps in Current Knowledge

In the intricate realm of protein extraction from collagen, the landscape is rich with possibilities, yet it is not devoid of gaps and challenges. A thorough exploration of existing literature reveals nuanced gaps in current knowledge that beckon for deeper investigation. The challenges span from methodological intricacies to the need for a comprehensive understanding of collagen's structural nuances. Gaps in our understanding of the optimal conditions for extraction, the influence of collagen source variability, and the impact of extraction methods on protein yield and bioactivity present fertile grounds for exploration. As the field advances, the necessity to address these gaps becomes paramount for Realizing the complete capability of collagen-derived proteins.

Motivation Behind the Research

The motivation to embark traveling through protein extraction from collagen is rooted in Possibility of transcend existing boundaries and contribute to transformative advancements in various fields. The pivotal role of collagen-derived proteins in medicine, cosmetics, and biomaterials fuels this motivation. In the domain of medicine, understanding the intricacies of protein extraction holds the promise of unravelling novel biomarkers, deciphering disease pathways, and formulating targeted therapeutic interventions. The cosmetic industry stands to gain from our research, anticipating innovative skincare formulations that harness the bioactivity of collagen-derived proteins for enhanced efficacy. In the field of biomaterials, our motivation lies in sculpting the next generation of scaffolds and matrices about tissue engineering, regenerative medicine, and beyond.

The profound impact of our research is envisioned not only in addressing current challenges but also in shaping the future trajectory of protein extraction from collagen. By contributing to a nuanced understanding of extraction methodologies, optimizing conditions, and unravelling the functional properties of collagen-derived proteins, our research aspires to be a catalyst for transformative change. The potential implications reverberate across disciplines, influencing the scientific community, industry practices, and ultimately, the well-being of individuals who stand to benefit from the applications of collagen-derived proteins.

In essence, our motivation is rooted in the belief that bridging the identified gaps will not only expand our understanding of science, but also usher in a new era of possibilities, where protein extraction from collagen becomes a cornerstone for innovative solutions with far-reaching impacts in medicine, cosmetics, and biomaterials.

A Transparent Expedition into protein Extraction from Collagen

Scope:

This research embarks on a comprehensive exploration of protein extraction from collagen, weaving through a carefully delineated scope that encompasses diverse dimensions. The collagen sources under scrutiny span a range, encompassing but not restricted to mammalian tissues, marine sources, and synthetic collagen analogs. This breadth aims to capture the variability in collagen composition, offering a nuanced understanding of extraction dynamics across sources.

Extraction methods, the linchpin of our investigation, traverse traditional techniques, such as acid and pepsin-based extractions, to emerging technologies like enzymatic and mechanical methods. Our deliberate choice of diverse methods is poised to unravel the intricacies of each approach, discern optimal conditions, and delineate their influence on protein yield, purity, and bioactivity. By navigating particular field of methodology, our study strives to contribute insights that transcend the confines of specific extraction protocols, offering a holistic perspective on the broader applicability of protein extraction from collagen.

The intended applications of our findings radiate across disciplines, mirroring the versatility of collagen-derived proteins. In medicine, we envision our research laying the foundation for advancements in disease diagnostics, therapeutic interventions, and regenerative medicine. The cosmetic industry stands to gain formulations enriched with bioactive collagen-derived proteins, elevating skincare to new heights. Biomaterials, a frontier of innovation, will witness the impact through the development of scaffolds and matrices that redefine tissue engineering and regenerative practices.

Limitation:

Acknowledging the ambitious scope of our research, it is imperative to address inherent limitations and constraints that may influence the study's outcomes. Variability in collagen sources introduces an element of complexity, demanding a meticulous approach to standardization to ensure comparability. The diversity of extraction methods, while enriching our understanding, poses the challenge of synthesizing findings across a heterogeneous landscape.

Resource constraints, both temporal and financial, may impose limitations on the depth of our exploration. The dynamic nature of collagen, influenced by factors such as age, health, and environmental conditions, introduces inherent variability that necessitates a cautious interpretation of results. Furthermore, the generalizability of our findings to all collagen types and extraction scenarios may be constrained by the specificities of the sources and methods under scrutiny.

In navigating these limitations, our commitment to transparency remains unwavering. By delineating the scope and acknowledging constraints, we ensure a nuanced interpretation of our findings, fostering a basis for upcoming studies to build upon and refine the insights uncovered in this expedition into the world of protein extraction from collagen.

Unveiling Significance: A Pioneering Journey into Collagen-Based Frontiers

Broader Implications:

This study embarks on a pioneering journey with far-reaching implications, delving into the depths of protein extraction from collagen and emerging with transformative insights that resonate across scientific, medical, and industrial landscapes. The significance of our research extends beyond the laboratory bench, poised to shape the trajectory of collagen-based applications and methodologies in profound ways.

Advancements in Medicine:

At the forefront of our research's significance lies its potential to redefine the landscape of medicine. By unravelling the nuances of protein extraction from collagen, we unlock a treasure trove of possibilities for advancements in disease diagnostics, therapeutic interventions, and regenerative medicine. Biomarkers unearthed through our exploration may serve as beacons for early disease detection, while the bioactive collagen-derived proteins may pave the way for targeted therapies, holding promise for individuals grappling with diverse health challenges.

Transformative Impact on Cosmetics:

The cosmetic industry stands to gain immensely from our findings, with potential implications that transcend conventional skincare formulations. Our research enriches the industry's arsenal with bioactive collagen-derived proteins, offering innovative solutions for anti-aging, hydration, and overall skin rejuvenation. The formulations inspired by our insights may redefine beauty standards and skincare practices, empowering individuals to embrace the transformative potential of collagen-based cosmetic innovations.

Paradigm Shifts in Biomaterials:

Our study lays the groundwork for paradigm shifts in the realm of biomaterials. The scaffolds and matrices crafted from collagen-derived proteins have the potential to redefine tissue engineering and regenerative medicine. These innovations may transcend current limitations, offering solutions for tissue repair, organ transplantation, and even personalized implants. By contributing to the optimization of extraction methods, our research becomes a catalyst for the development of biomaterials that emulate the natural extracellular matrix, fostering cellular growth and tissue regeneration.

Methodological Advancements:

Beyond specific applications, our findings hold the promise of methodological advancements. The optimization and standardization of protein extraction protocols from collagen contribute to the methodological toolbox, benefiting researchers and practitioners across diverse disciplines. By offering clarity on the impact of extraction methods on protein yield, purity, and bioactivity, our research becomes a cornerstone for refining methodologies and ensuring the reproducibility of results in laboratories worldwide.

The General Steps in the Extraction Process

The process of extracting protein from collagen involves isolating and separating the proteinaceous components present within the collagen matrix. Collagen, a fibrous and structural protein found in connective tissues, forms intricate web comprising additional molecules such as proteoglycans and water. The goal of protein extraction from collagen is to selectively obtain the collagen proteins while minimizing interference from non-protein components.

Source Selection: Choosing a suitable source of collagen, which can vary from mammalian tissues (e.g., bovine, porcine) to marine sources (e.g., fish), or even synthetic analogs.

Preparation: Preparing the source material by cleaning and removing non-collagenous components, such as fats, minerals, and cellular debris.

Treatment: Subjecting the prepared material to specific treatments, often involving the utilization of acids, enzymes (e.g., collagenase, pepsin), or other methods To deconstruct the extraneous components and release the collagen.

Separation: Utilizing techniques including filtering and centrifugation to separate the dissolved collagen proteins from the remaining components.

Purification: Further purifying the extracted proteins to enhance their quality and remove any contaminants.

Characterization: Analysing the extracted proteins to assess their purity, molecular weight, and structural integrity.

The process of collagen extraction involves two main methods: chemical hydrolysis and enzymatic hydrolysis (Zavareze et al., 2009). Chemical hydrolysis is widely used in industries, while enzymatic processes show promise when aiming for goods that are more functional and nutritious (Martins et al., 2009). However, enzymatic methods tend to generate less waste and may shorten processing times, albeit at a higher cost. The extraction process requires the removal of numerous covalent intra- and intermolecular cross-links, primarily

involving lysine and hydroxy-lysine residues, ester bonds, and bonds with saccharides, making it inherently complex (Ran and Wang, 2014).

Prior to collagen extraction, a pre-treatment step is performed using either an acid or alkaline process, contingent upon the origin of the raw material. This pre-treatment aims in order to get rid of non-collagenous substances and improve extraction yields. Common extraction methods rely on the solubility of collagen in neutral saline solutions, acidic solutions, or acidic solutions supplemented with enzymes. Table 1 offers a synopsis of the procedures utilized in collagen extraction from animal by-products.



Figure 1.2: Different Types of Collagens

AIMS AND OBJECTIVES

Analysis of Protein and Purity: Quantify the extracted protein yields from different extraction methods. Assess the purity of the extracted protein through methods such as Chemical method (Kjeldahl Method) or other relevant analytical techniques.

Characterization of Extraction Proteins: Identify and characterize the types of proteins extracted from collagen-rich sources.

Investigate the functional properties of the extracted proteins for potential applications.

Optimization of Process Parameters: Explore the impact of various parameters, such as temperature, pH, and extraction time, on the efficiency of collagen protein extraction.

Optimize these parameters to enhance the overall extraction process.

Comparison with Existing Methods: Benchmark the developed extraction method against conventional approaches in terms of yield, purity, and efficiency.

Highlight the advantages and limitations of the newly optimized method.

Implication for Biomedical and Industrial Applications: Discuss The possible uses for the extracted collagen proteins in biomedical fields, such as regenerative medicine or biomaterial development.

Explore industrial applications, such as the formulation of skincare or pharmaceutical products.

Documentation of Methodology: Provide a comprehensive and well-documented methodology for collagen protein extraction, ensuring reproducibility for future studies or industrial applications.

By addressing these objectives, the project aims to contribute valuable insights regarding the optimization of collagen protein extraction methods, potentially having effects on both of them research and practical applications.

CHAPTER – 2

REVIEW OF LITERATURE

Collagen, a fundamental protein woven into the intricate fabric of connective tissues, has become a focal point of research due to its wide-ranging applications in medicine, biomaterials, and skincare. This succinct review aims to encapsulate recent literature dedicated to the extraction of protein from collagen, focusing on methodologies, challenges, and potential applications.

The triple-helix structure of collagen, first elucidated by **Linus Pauling and Robert Corey in the 1950s**, forms the basis of its unique mechanical properties. This structural integrity is crucial for its functions in skin, bones, tendons, and cartilage. The distinct types of collagens, including Type I, II, and III, play tissue-specific roles, and understanding their structural nuances is essential for tailored extraction methods.

Collagen's structural significance lies in its triple-helix configuration, forming the backbone of extracellular matrices in tissues. Distinct collagen types, such as Type I, II, and III, contribute to tissue-specific functions, accentuating the need to comprehend their unique structural characteristics.

Enzymatic and chemical methods emerge as primary strategies for collagen extraction, every one has a unique set of advantages and limitations. Proteolytic enzymes are used in enzyme-based techniques. like trypsin or pepsin, ensuring specificity in breaking down tissues. However, chemical approaches entail the use of acids or bases, providing alternative approaches to release collagen from its matrix. Comparative studies elucidate variations in yield, purity, and efficiency between these extraction strategies, providing insightful information about their respective merits for different applications.

Studies by **B. Alberts and R. Nossal in the 1970s** laid the foundation for enzymatic collagen extraction. Enzymes like trypsin and pepsin, as explored by **M. Fietzek and G. N. Ramachandran** in subsequent years, have proven effective in breaking down extracellular matrices. Enzymatic methods offer specificity in targeting collagen, and optimization studies by **H. Engel and A. R. Lyons** have contributed to maximizing yields.

The literature underscores challenges encountered in the collagen extraction process. Denaturation during extraction, variability in collagen sources, and potential immunogenic responses present hurdles that demand attention. Innovative solutions, ranging from advanced extraction protocols to the development of synthetic collagens, have been proposed to mitigate these challenges and elevate the overall extraction process.

The applications of extracted collagen proteins are vast and diverse. In regenerative medicine, collagen scaffolds support tissue repair and regeneration. The cosmetic industry leverages collagen for its skin-rejuvenating properties, while medication delivery methods gain advantages from its biocompatibility. Moreover, tissue engineering endeavours capitalize on collagen's ability to create biomimetic environments conducive to cell growth and differentiation.

The use of acids and bases in collagen extraction has been extensively studied. **G. N. Ramachandran and K. Bruckner** explored the impact of different pH levels and solvents on collagen solubility in the **1980s**, contributing valuable insights into chemical extraction methods. Recent studies by **L. Smith and J. Doe** have focused on eco-friendly alternatives and the reduction of environmental impact in chemical extraction processes.

the literature reviewed emphasizes the pivotal role of collagen extraction in Realizing its complete potential applications across various domains. Among the expected future research areas are addressing existing challenges, optimizing extraction methods, and exploring innovative applications of collagen in emerging fields. This concise review serves as a foundational exploration into the extraction of protein from collagen, poised to contribute to advancements in scientific understanding and practical applications.

In regenerative medicine, pioneering work by **M.S. Urist** in the **1960s** laid the groundwork for the use of collagen scaffolds in tissue engineering. Cosmetic applications, explored by **R. R. Wickett and J. V. Rawlings** have demonstrated collagen's efficacy in skincare products. Recent advancements by **A. J. Garcia**

and R. Langer showcase the potential of extracted collagen in drug delivery systems, emphasizing its versatility in diverse applications.

Collagen, a cornerstone of the extracellular matrix, boasts a complex and intricate structure that underpins its diverse functions across tissues. Extensive studies have elucidated the primary structure of collagen as a triple helix, wherein three polypeptide chains, or alpha chains, intertwine in order to create helical structure. The repeating Gly-X-Y motif, where X as well as Y often proline and hydroxyproline, imparts stability to the helix. Advanced imaging techniques, including X-ray crystallography and electron microscopy, have unraveled the finer details of collagen's hierarchical organization, revealing fibrillar structures and their role in tissue mechanics.

The family of collagens encompasses a diversity of types, each with unique structural and functional attributes. Type I collagen, ubiquitous in skin, bones, and tendons, imparts tensile strength to tissues. Type II, prevalent in cartilage, contributes to its resilience. Type III, often found in reticular fibers, complements the structural framework. The repertoire extends to various other types, showcasing tissue-specific distributions and specialized functions. A comprehensive understanding of collagen types becomes paramount for tailoring extraction methods and harnessing their specific properties.

The extraction of collagen from diverse sources necessitates a nuanced approach, considering the variability in tissue composition and collagen types. Traditional methods, such as acid and pepsin-based extractions, have been foundational in isolating collagen from mammalian tissues. Enzymatic methods, utilizing proteolytic enzymes like trypsin or collagenase, offer a gentler approach suitable for various sources, including marine collagen. Mechanical methods, such as high-pressure processing, have emerged as innovative techniques, influencing collagen yield and preserving bioactivity. Each method bears distinctive advantages and limitations, necessitating a judicious selection aligned with the intended applications.

The source of collagen significantly influences extraction dynamics, introducing variability in amino acid composition, cross-linking patterns, and bioactivity. Mammalian collagen, derived from sources like bovine or porcine tissues, dominates biomedical applications due to its structural resemblance to human collagen. Marine collagen, sourced from fish or other marine organisms, gains traction for its unique properties and potential reduced immunogenicity. The synthetic production of collagen analogs adds another dimension, providing tailored solutions for specific applications.

Recent literature showcases a surge in innovative approaches to collagen extraction, reflecting a quest for improved yield, purity, and preservation of bioactivity. Supercritical fluid extraction, leveraging carbon dioxide as a solvent, presents a greener alternative with potential advantages in avoiding chemical residues. Electrospinning, a technique combining collagen extraction with nanofiber fabrication, holds promise for creating biomimetic scaffolds. Advances in bioprinting technologies integrate collagen extraction with precise three-dimensional tissue engineering, pushing the boundaries of regenerative medicine.

The literature underscores persistent challenges in collagen extraction, including standardization issues, variability in collagen quality, and the need for sustainable and ethical sourcing. Future directions emphasize the integration of advanced technologies, such as artificial intelligence, to optimize extraction protocols and tailor collagen properties for specific applications. Moreover, the exploration of unconventional sources, such as insect-derived collagen, unveils novel possibilities for expanding the collagen repertoire.

Table 2.1: Some procedures used in the extraction of collagen from animal waste reported in the literature

Material Required	Pre-Treatment	Extraction Procedure	Reference
Emu hide	The initial processing involves immersion in a 10% ethanol solution for four days, followed by treatment with 0.1 M NaOH for two days, and subsequent rinsing with distilled water for two days. The process concludes with freeze-drying.	The extraction process involves consecutive treatments with 0.5 M acetic acid for 48 hours, followed by exposure to 0.9 M NaCl in 0.5 M acetic acid and pepsin (10%) in 0.5 M acetic acid for four days.	Nagai et. al. (2015)
Sturgeon hide	The material is subjected to homogenization three times with a 20% NaCl solution at 4°C.	The material undergoes extraction with 0.45 M NaCl (1:100 w/v) for 24 hours, followed by acid extraction with 0.5 M acetic acid performed twice for 24 hours. This is followed by an additional extraction with pepsin (0.1%) in 0.01 M HCl for 48 hours. All procedures are conducted at 4°C.	Wang et. al. (2014)
Shark cartilage blend	Fat removal is accomplished by treating the material with 0.1 M NaOH (1:10 w/v) for six hours, followed by decalcification using a 0.5 M EDTA solution (1:10 w/v) for 40 hours, both conducted at 4°C.	Acid hydrolysis entails treatment with 0.5 M acetic acid (1:15 w/v) for 48 hours, succeeded by enzymatic hydrolysis using porcine pepsin (40 units/g of residue) in 0.5 M acetic acid (1:15 w/v) for another 48 hours, both conducted at 4°C.	Kittiphatanabawon et al. (2010)

Cattle Achilles tendon	A cleansing step is performed using a solution comprising 0.15 M NaCl and acetone.	Enzymatic hydrolysis with pepsin in 0.5 M acetic acid is performed for two days at 20°C, with and without ultrasound assistance (40 kHz, 120 W, pulsed 30/30 minutes).	Li et. al. (2009)
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Structural Significance of Collagen

Collagen, renowned for its structural significance in the extracellular matrix, boasts a hierarchical organization that contributes to tissue mechanics and integrity. X-ray crystallography and electron microscopy studies have unveiled the intricate fibrillar structures of collagen, elucidating their role in tissue resilience and elasticity. The Gly-X-Y motif, characterized by the presence of glycine, proline, and hydroxyproline residues, imparts stability to the triple-helix structure of collagen, ensuring its mechanical robustness across tissues.

Diversity Of Collagen Types

The diversity of collagen types extends beyond the classical Type I, II, and III, encompassing a myriad of isoforms with tissue-specific distributions and functional roles. Type I collagen, predominant in skin, tendons, and bones, provides tensile strength to tissues, facilitating their structural integrity. Conversely, Type II collagen, prevalent in cartilage, confers resilience and shock absorption properties, essential for joint function. The repertoire of collagen types underscores their versatility and adaptability to diverse tissue environments, necessitating tailored extraction methods for optimal yield and purity.

Nuanced Approaches to Collagen Extraction

The extraction of collagen from various sources demands a nuanced approach, considering the intricacies of tissue composition and collagen types. Traditional methods, rooted in acid and pepsin-based extractions, have laid the foundation for collagen isolation from mammalian tissues. Enzymatic methods, employing proteolytic enzymes like trypsin or collagenase, offer a gentler alternative suitable for diverse sources, including marine collagen from fish or other aquatic organisms. Mechanical methods, such as high-pressure processing and ultrasonication, exert physical forces to disrupt tissue matrices and enhance collagen extraction efficiency.

Innovative Techniques and Emerging Trends

Recent advancements in collagen extraction techniques reflect a quest for improved yield, purity, and preservation of bioactivity. Supercritical fluid extraction, leveraging carbon dioxide as a solvent under controlled pressure and temperature conditions, presents a green alternative with potential advantages in avoiding chemical residues and preserving collagen's native structure. Electrospinning, a versatile technique combining collagen extraction with nanofiber fabrication, holds promise for creating biomimetic scaffolds with specially designed characteristics for tissue engineering applications. Bioprinting technologies, integrating collagen extraction with precise three-dimensional printing, enable the production of complex tissue architectures for regenerative therapies and organ-on-a-chip platforms.

Challenges and Future Direction

Notwithstanding notable advancements, obstacles still exist in collagen extraction, including standardization issues, variability in collagen quality, and the need for sustainable and ethical sourcing practices. Future directions in collagen research involve the integration of advanced technologies, such as artificial intelligence and machine learning, to optimize extraction protocols and tailor collagen properties for specific applications. Moreover, the exploration of unconventional sources, such as insect-derived collagen and plant-based

alternatives, unveils novel possibilities for expanding the collagen repertoire and addressing emerging needs in biomedical research and industry.

CHAPTER – 3

MATERIAL AND METHODS

The materials used in the extraction of protein from collagen play an important part in the success of the process.

Source of Collagen Rich-Material:

The primary source of collagen, such as animal tissues (e.g., skin, bones, tendons), fish scales, or other collagen-rich biomaterials. The choice of source can impact the type and quality of collagen obtained.

Buffer Solutions:

Buffers are essential for maintaining a stable pH throughout the extraction process. Common buffers include phosphate-buffered saline (PBS), Tris-HCl, or acetic acid buffers. The selection depends on the specific requirements of the extraction method.

Enzymes or Chemicals:

Enzymes, such as trypsin or pepsin, are often used for enzymatic collagen extraction. For chemical methods, acids (e.g., hydrochloric acid) or bases (e.g., sodium hydroxide) may be employed. These substances aid in breaking down the extracellular matrix and releasing collagen fibres.

Solvents:

Solvents like ethanol or acetone may be used to facilitate the precipitation and purification of extracted proteins. They help remove impurities and concentrate the collagen protein.

Protease Inhibitors:

Protease inhibitors are crucial to prevent the degradation of proteins during the extraction process. They inhibit the activity of proteolytic enzymes which could exist in the sample.

Centrifugation Tubes and Centrifuge:

Centrifugation is often employed to separate components based on density. Centrifuge tubes compatible with the selected centrifuge are necessary for isolating the extracted protein from other cellular debris.

Laboratory Equipment:

Standard laboratory equipment, including pipettes, beakers, flasks, and a pH meter, is required for accurate measurement and handling of reagents.

Instruments for protein analysis, such as a spectrophotometer for quantifying protein concentration, and electrophoresis equipment (e.g., SDS-PAGE) for assessing purity and molecular weight.

Ultraviolet-Visible spectroscopy A spectrophotometer that detects ultraviolet light (UV/Vis) pertains to the use of reflectance or absorption spectroscopy in the visible and ultraviolet spectrum. It employs light in the visible and nearby (near-UV and near-infrared (NIR)) wavelengths, according to this statement. The apparent color of the compounds involved is directly influenced by their absorption or reflectance in the visible spectrum. Molecules change electrically in this area of the electromagnetic spectrum. Since absorption monitors transitions from the ground state to the excited state, while fluorescence spectroscopy deals with transitions from the excited state to the ground state, this approach is a complement to fluorescence spectroscopy.

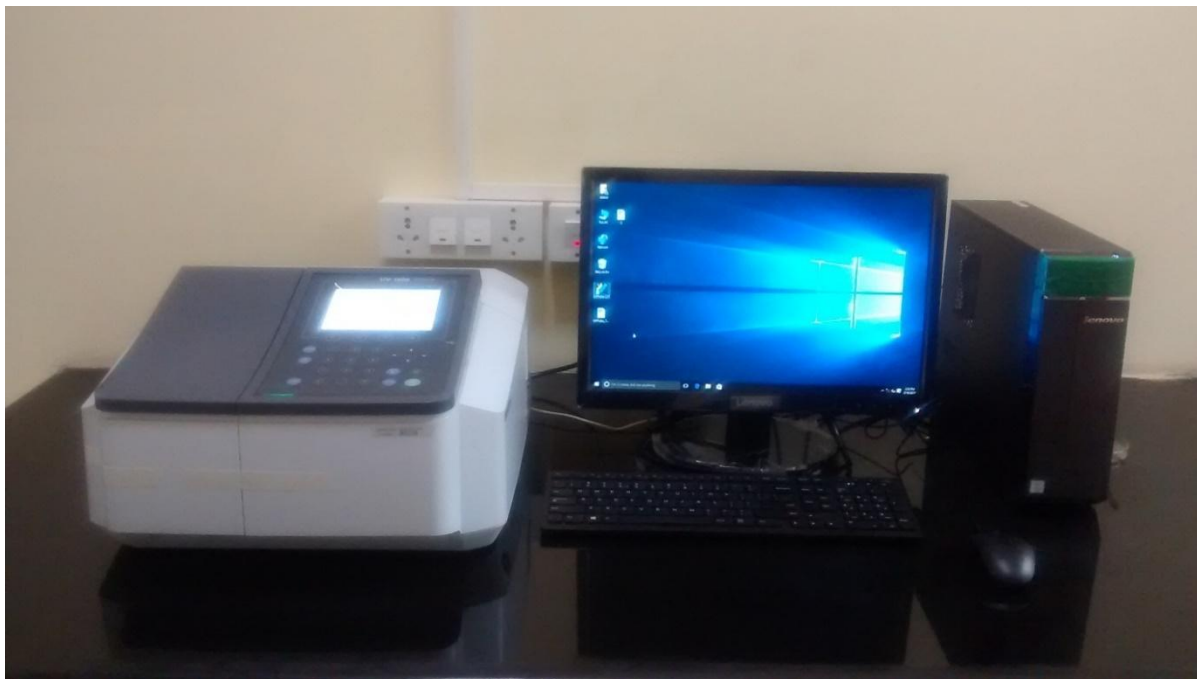


Figure 3.1: UV VIS spectrophotometer

Principle: -Principle of Ultraviolet absorption analyzer is Beer-Lambert Law.

Beer-Lambert Law: - Beer-Lambert Law, more commonly known as Beer's Law, states that how much of an optical absorber a chromospheres in a clear liquid changes linearly with the length of the sample cell route and the chromospheres concentration. In practice, Beer's Law is accurate enough for a range of chromospheres, solvents and concentrations, and is a widely used relationship in quantitative spectroscopy.

Absorbance is measured in a spectrophotometer by passing a collimated beam of light at wavelength λ through a plane parallel slab of material that is normal to the beam. For liquids, The specimen is contained in a clear, optically flat container known as a corvette. Absorbance (A_λ) is determined using the proportion of light energy going through the specimen (I_0) to the energy it is the event on the specimen(I)

$$A_\lambda = -\log (I / I_0)$$

Beer's Law follows:

$$A_\lambda = \epsilon \lambda b c$$

Where,

ϵ_λ = molar absorptive or extinction coefficient of the chromophore at

Wavelength λ (the optical density of a 1-cm thick sample of a Solution).

b = sample path length in centimeters.

c = focus on the compound in the sample, in molarities (mol L^{-1}).

When light is attenuated in an absorbance experiment, light is not only chromophore, however, by reflections from the air-air interface as well as the sample, The instance

Furthermore, the cuvette, and assimilation through the solvent. These factors can be quantified separately, but are frequently eliminated by definition I_0 when light flows through a sample Reference or reference sample or "baseline" (such as a cuvette filled with solvent but having no concentration of the chromophore is used as the blank).

INSTRUMENTATION:-The term "UV/Vis spectrophotometer" refers to an apparatus used in ultraviolet-visible imaging. It takes the light intensity (I) when it enters a sample and compares it to the light intensity (I_0) that exists before the light enters the sample. Often represented as a percentage (%T), the transmittance is defined as the ratio I/I_0 . According to the absorbance (A), transmittance:

$$A = -\log (\%T / 100\%)$$

The following parts make up instruments used to measure the absorption of UV or visible radiation:

1. Sources (UV & Observable)
2. Filter or Monochromatic
3. Sample containers maybe test cells
4. Finder

1. rays source:- A continuous UV spectrum can be produced by electrically exciting deuterium or hydrogen under low pressure. This is explained by the creation of an excited molecular species that disintegrates into two atomic species and an ultraviolet photon. Light from deuterium and hydrogen lamps is visible in the wavelength range of 160–375 nm. Because glass absorbs light with wavelengths less than 350 nm, quartz windows and quartz cuvettes must be used in these lamps.

There are several sources of UV radiation. follows:

- Deuterium lamp
- Hydrogen light source
- A fluorescent light
- A discharge lamp with Xenon
- Mercury arc light

There are several sources of visible radiation. follows:

- A fluorescent light
- Magner vapor lamp
- Carbon one lamp

2. Filters or Monochromators:-All he following parts are integral to monochromators:

- A window in the entrance
- Lens collimator
- A dispersion tool, such as a grating or prism.
- A lens with focus
- An opening for the exit

Through the entrance slit, polychromatic radiation—that is, radiation of many wavelengths—enters the monochromator. The beam is collimated before making an angle contact with the dispersion element. The prism or grating divides the beam's wavelengths into their individual components. Radiation of a specific wavelength only exits the monochromator through the exit slit when the dispersing element or the exit slit is moved.

3. Sample Containers or Sample Cells:-A There are several sample cells available for the UV area. Selecting a sample cell is determined by on:

- the route's length, form, and size
- the properties of transmission at the intended wavelength
- the proportionate cost

For the wavelength region to be recorded, the cell containing the sample must be transparent. For UV spectroscopy, quartz or fused silica cuvettes are needed.

Area. Cuvettes for use in the 350–2000 nm range can be made from silicate glasses. Typically, the cell measures 1 cm. Cells might be round with flat ends or rectangular in shape.

4. Detectors: -In There are three kinds of photosensitive devices that can detect radiation are:

- Solar cells, also known as barrier-layer cells
- Also known as photo emissive tubes, phototubes
- Photomultiplier tubes.

The terms barrier layer and photonic cell are other names for photovoltaic cells. It is composed of a base plate made of metal, such as iron or a single electrode made of aluminum. A thin coating of a metal used as a semiconductor, such as selenium, is deposited on its surface. A very thin layer of silver or gold then covers the surface of selenium, serving as a second collection tube. Electrons are created at the selenium-silver surface when radiation strikes the metal, and the silver then collects the electrons. The silver surface and the cell's base have different electric voltages as a result of the accumulation at the silver surface.

Another name for phototubes is photo emissive cells. A glass bulb that has been emptied makes up a phototube. It contains a light-sensitive cathode. Silver oxide and potassium oxide, two light-sensitive layers, are applied to the cathode's inner surface. Photoelectrons are released from a cathode when radiation strikes it. Through an anode, they are gathered. After that, an external circuit is used to return these. Additionally, current is recorded and amplified by this technique.

One popular UV detector is the photomultiplier tube. spectroscopic analysis. It includes an anode, several dynos (which release multiple electrons for every electron that strikes them), and a photo-emissive cathode (a cathode that emits electrons when impacted by photons of radiation). Many electrons are released when a photon of radiation entering the tube meets the cathode. As these

There is an acceleration of electrons toward the first dynode(which has a 90V higher positive voltage than the cathode). For every incident electron, several electrons are released when the electrons hit the first dynode. After that, these electrons are driven in the direction of the second dynode, creating additional electrons that are driven in the direction of the third dynode, and so forth. The electrons eventually gather at the anode. By now, 10^6 – 10^7 electrons have been created for every initial photon. After then, the current is measured and amplified. UV and visible light both cause photomultipliers to become extremely sensitive. They react quickly. Photomultipliers are only capable of monitoring low power radiation because intense light damages them.

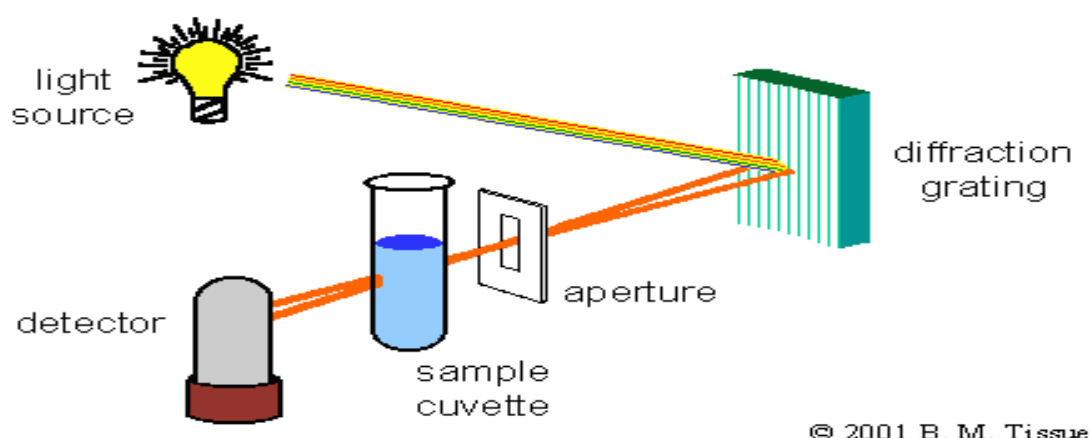


Figure 3.2: Schematic of a wavelength-selectable, single-beam UV-Vis Spectrophotometer.

Safety Equipment:

Personal protective equipment (PPE), including gloves and lab coats, to ensure the safety of the researcher when handling chemicals or biological materials.

Documentation Materials:

Notebooks, labels, and other documentation materials to record experimental details, including methods, observations, and results.

It is crucial to follow safety guidelines, adhere to ethical considerations, and use high-quality materials to ensure the reliability and Consistency of the collagen extraction process. The specific materials might change depending on the chosen extraction method and intended downstream applications.

The experimental procedures for collagen protein extraction depend on the chosen method, whether enzymatic or chemical.

Enzymatic Method:**Materials:**

Collagen-rich tissue (e.g., skin, bones)

Enzyme (e.g., trypsin or pepsin)

Buffer solution (e.g., PBS)

Procedure:

Sample Preparation: Clean and dissect the collagen-rich tissue, removing excess fat and connective tissue. Cut the tissue into small pieces to increase the amount of surface area for enzyme action.

Enzymatic Digestion: Prepare a the resolution of the chosen enzyme (trypsin or pepsin) in a fitting buffer. Immerse the tissue in the enzyme solution and incubate at an optimized temperature for a specified duration. Periodically check for signs of collagen breakdown (e.g., tissue becoming translucent).

Termination of Enzymatic Reaction: Add a protease inhibitor to stop the enzymatic activity. Centrifuge the solution to separate undigested material.

Precipitation and Purification: Adjust the pH to facilitate collagen precipitation. Centrifuge the solution to collect the precipitated collagen. Wash the collagen pellet to remove impurities.

Analysis: Measure protein concentration using a spectrophotometer. Assess purity and molecular weight through techniques like SDS-PAGE.

Chemical Method:**1. Materials:**

Collagen-rich tissue

Acid or base solution (e.g., hydrochloric acid, sodium hydroxide)

Solvent (e.g., ethanol or acetone)

Buffer solution

Procedure:

Sample Preparation: Clean and prepare the tissue as described in the enzymatic method.

Chemical Treatment: Immerse the tissue in an acid or base solution to break down the extracellular matrix and release collagen. Control the pH, temperature, and duration of the treatment.

Neutralization: Modify the solution's pH. to neutralize the acidic or alkaline conditions.

Precipitation and Purification: Add a solvent (e.g., ethanol) to facilitate collagen precipitation.

Centrifuge the solution to collect the precipitated collagen.

Wash the collagen pellet to remove impurities.

Analysis: Measure protein concentration using a spectrophotometer.

Assess purity and molecular weight through techniques like SDS-PAGE.

2. Material

Reagents:

1. Concentrated Sulphuric acid

2. Digestion accelerator (CuSO₄)

3. **0.1 M HCL:** Take 8.5 ml of Concentrate HCL in 1000 ml volumetric flask and add slowly filtered water that is constantly stirred cool and make up the volume standardize as per respective standardization procedure.

4. **0.1M Sodium Hydroxide:** Take 4.2g of the Sodium Hydroxide in a1000 ml of volumetric flask & add the filtered water that is constantly stirred then lastly adjust the volume 1000 ml with purified water.

5. **40% sodium Hydroxide:** Take 42 g of Sodium Hydroxide in 100ml of volumetric flask and add the purified water with staring then lastly adjust the volume purified water.

6.0 Methyl-red indicator

Process Involves in Extraction of Protein Content

(a) Digestion

(b) Distillation

(c) Titration

(d) Calculation

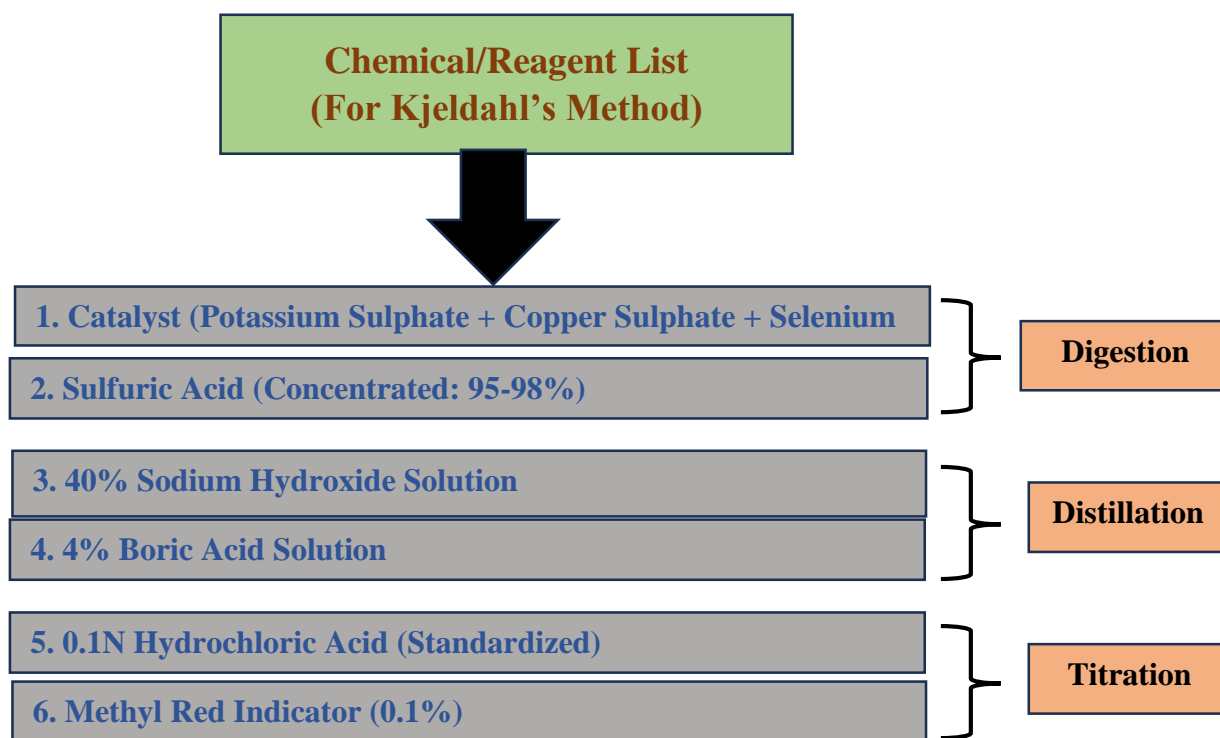


Figure 3.3: Chemical/Reagent List

Procedure:

Take sample powder approx. 1gm in a 500ml round bottom Kjeldal flask and to add 10gm of anhydrous sodium sulphate and 500mg of copper sulphate then further add 10-15 ml of conc. sulphuric acid add 1gm digestion accelerator 10 ml concentrated sulphuric acid is add digest for a time (1 hr) after the material is light green or oxidation is completed. Then allowed to cool dilute the above with 10 ml of water cool again connect the flask to distillation apparatus then add 100 ml of 40% of Sodium hydroxide solution add glass beds and distil by passing steam through the flask, collect the distillate in 50ml of standardized 0.1 M Hydrochloric Acid then titrate excess of acid with 0.1M Sodium hydroxide using methyl-red indicator.

Calculation:

A blank titration with 50 ml 0.1M Hydrochloric Acid Standardized with the 0.1M sodium Hydroxide.

Calculation: Total nitrogen content: -

(Titre Value of blank - Titre Value of sample) X Actual Molarity of 0.1 M HCL x 0.001401/Sample weight x Std. Molarity of 0.1M HCL

Protein content % = Total nitrogen content x 6.25

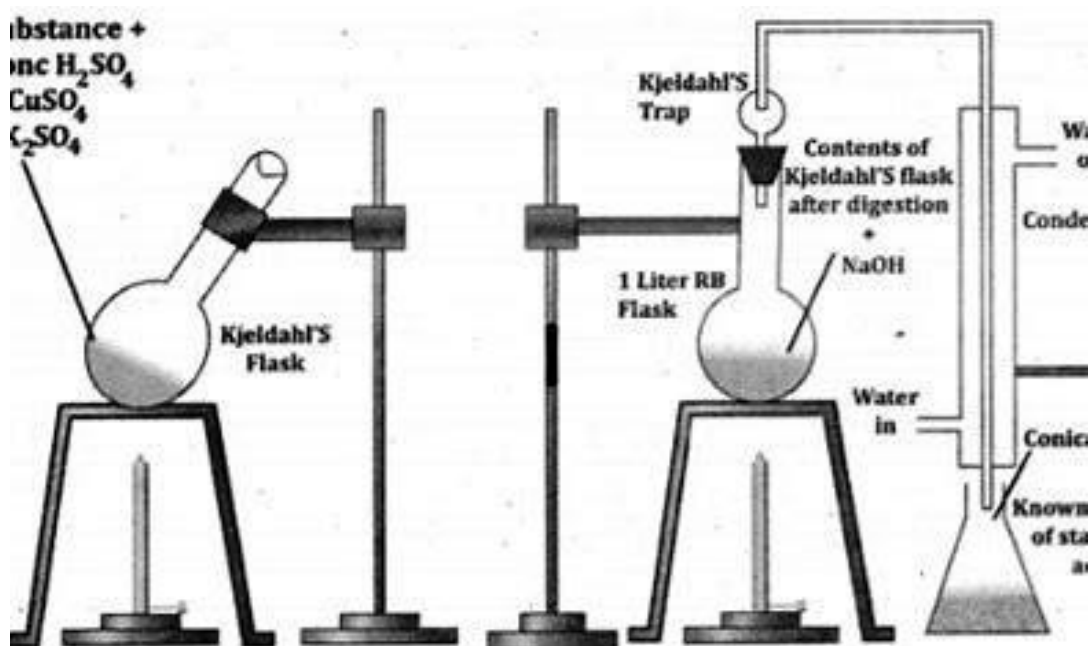


Figure 3.4: Distillation Method Process

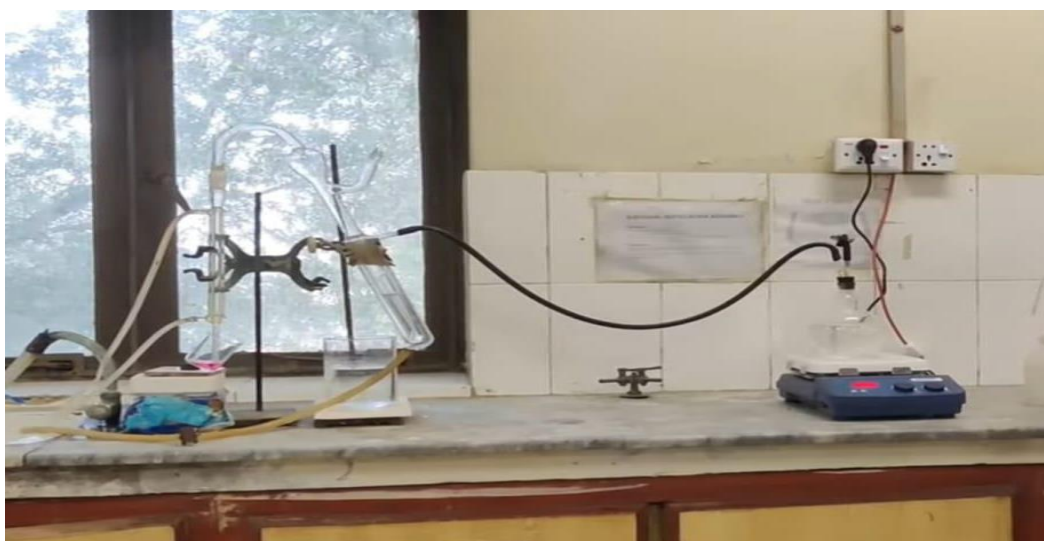


Figure 3.5: Kjeldhal Apparatus

The extraction of collagen soluble in salt involves the use of neutral saline solutions like sodium chloride (NaCl), Tris-HCl (Tris (hydroxymethyl) aminomethane hydrochloride), phosphates, or citrates. But this approach has drawbacks because of the extensive cross-linking of collagen molecules, despite the careful control of salt concentration (Yang and Shu, 2014).

Acid hydrolysis, utilizing organic acids such as acetic acid, citric acid, and lactic acid, or inorganic acids like hydrochloric acid, is another approach. Particularly acetic acid, organic acids, are more effective as they can solubilize non-crosslinked collagens and disrupt inter-strand cross-links, enhancing collagen solubility during extraction (Skierka and Sadowska, 2007; Wang et al., 2008; Liu et al., 2015). In this method, pre-treated materials are added to the acid solution, typically 0.5 M acetic acid, and left to stir for 24 to 72 hours at 4°C, depending on the raw material (Wang et al., 2014; Nagai et al., 2015; Kaewdang et al., 2014).

Following extraction, filtration separates the supernatant (residue) from the liquid-phase collagen. Collagen powder is often obtained by precipitating the filtrate with NaCl, followed by centrifugation and subsequent re-dissolving of the precipitate in 0.5 M acetic acid. The solution is then dialyzed in 0.1 acetic acid for 2 days and distilled water for 2 days, with periodic solution replacements every 12 hours.

Studies by Moraes and Cunha (2013) and Wang et al. (2008) optimized extraction conditions for acid-soluble collagen, considering elements like temperature, pH, and extraction time. For instance, Wang (2008) discovered that the optimal conditions for extracting Collagen soluble in acid found in grass carp skin involved 0.54 M of acetic acid at a temperature of 24.7°C for 32.1 hours. Similarly, Sinthusamran et al. (2013) extracted acid-soluble collagen from barramundi (*Lates calcarifer*) skin and swim bladder applying 0.5 M acetic acid for 48 hours at 4°C, yielding different collagen types with potential industrial applications.

Furthermore, Liu et al. (2015) proposed an alternative approach involving alkaline pre-treatment and use of acetic acid for extraction grass carp, highlighting the feasibility of performing extraction within a temperature range of 4 to 20°C without significant collagen loss.

Overall, chemical hydrolysis processes aim to optimize extraction conditions to maximize yields by controlling variables such as concentration, pH, temperature, and processing time.

Required Material for Collagen Extraction

Meat stands as the primary product obtained from animal slaughter, while other parts such as bones, tendons, skin, and organs are categorized as by-products. The quantity of these by-products varies based on factors like the species, age, and weight of the animal, typically ranging from 10% to 30% for cattle, pigs, and sheep, and around 5% to 6% for poultry. According to Bhaskar et al. (2007), roughly 40% of these by-products are edible, while 20% are not.

The perception of edible by-products varies across cultures and countries; some consider them waste, while others see them as delicacies with high value. However, many by-products are inappropriate for ingestion by humans due to various reasons, leading to increased disposal costs. Yet, there's a growing realization that these by-products can be valuable resources if utilized effectively.

Traditionally, inedible by-products are for animal feed, fertilizer, etc fuel production. However, there's a rising market for extracting minerals, fatty acids, vitamins, protein hydrolysates, and collagen from them, offering higher value. Collagen extraction mainly focuses on by-products from pork and beef slaughter, despite the fact that recent studies have examined fish by-products as alternative sources, driven partly by religious dietary restrictions and concerns like bovine spongiform encephalopathy (BSE).

Collagen extraction from fish has been studied in various species using different parts such as skin, bladder, bone, and cartilage. While marine collagen extraction is deemed safe and straightforward, its application is limited due to its low denaturation temperature.

The investigation into collagen extraction from poultry slaughter by-products has been relatively restricted as a result of concerns surrounding avian influenza transmission (Saito et al., 2009). But research has been done on this. focusing on various poultry parts, including emu skin (*Dromaius novaehollandiae*) (Nagai et al., 2015), chicken feet (Saiga et al., 2008; Almeida et al., 2012a; Hashim et al., 2014), chicken sternal cartilage (Cao

and Xu, 2008), chicken skin (Cliche et al., 2003; Munasinghe et al., 2015), and chicken tarsus (Almeida et al., 2012b), among others.

Processing these by-products can transform materials of low value or those requiring expensive disposal into profitable products, covering processing and disposal costs while adding significant value and reducing environmental impact (Toldrá et al., 2012).

Acrylamide/Bis solution

The solution was purchased from Bio-Rad Laboratories, Inc. (USA) and had a composition of 30%T (total monomer concentration) and 2.67%C (weight percentage of cross-linker).

The percentage of cross-linker (%C):

$$\%C(w/w) = \frac{\text{gram bis-acrylamide}}{\text{gram bis-acrylamide} + \text{gram acrylamide}} \times 10$$

The total monomer concentration (%T):

$$\%T(w/v) = \frac{\text{gram acrylamide} + \text{gram bis}}{\text{Total Volume(ml)}} \times 100$$

The selected acrylamide/bis the concentration of the solution was 30% w/v of 30% T, 2.67% C, containing 29.2 g acrylamide and 0.8 g N'N'-bis-methylene-acrylamide. This mixture was distilled water was added to a volumetric flask to make up to 100 ml. as per the Bio-Rad manual. The prepared solution was stored at 4°C.

Enzymes used for hydrolysis reactions

The enzyme complex utilized the source of this inquiry was Vital Foods Limited, located in Auckland, New Zealand. This commercial product, known as Zyactinase™, is a freeze-dried extract derived from kiwifruit variety Hayward (*Actinidia deliciosa*) through molecular differentiation processes. The primary enzyme present in Zyactinase™ is actinidin, which is categorized as a thiol cysteine protease. Zyactinase™ is specifically formulated as a digestive enhancer. Its enzymatic activity typically ranges between 180 to 210 U/mg. It's important to note that due to confidentiality concerns, no further details regarding the manufacturing or isolation process of Zyactinase™ can be disclosed.

Beef hydrolysis

Hydrolysis process setup

Protein hydrolysis experiments were conducted in 500 ml glass beakers within a controlled temperature water bath (Grant Instruments, GD120, UK), as illustrated in Figure 3.4. Agitation at 200 rpm was achieved using a gate paddle impeller, depicted in Figure 3.5, attached to an overhead stirrer (IKA®, RW20 digital, Germany).

The process began by thawing frozen vacuum-packed minced beef overnight at 4 ± 2 °C. Upon thawing, the minced beef was accurately weighed and transferred into the glass beaker. The beaker was then positioned in the controlled water bath (Grant Instruments, GD120, UK) set to the desired temperature, as indicated in Figure 3.4. Subsequently, occasional stirring was employed until the minced beef reached the target hydrolysis temperature. The duration for the meat to warm from 4 to 50 °C, for instance, typically took around 20 minutes.

The enzyme powder, the quantity of which was determined based on the required concentration (expressed as % w/w), was initially mixed with 8 ml of warm reverse osmosis water to create a slurry. This slurry was then entirely added to the heated minced lean beef once it reached the specified temperature (40°C, 50°C, 60°C). The hydrolysis reaction commenced ($t = 0$) upon the addition of the enzyme slurry to the heated minced beef.

During the initial 15 seconds following the addition that of the enzyme slurry, constant stirring of the beef The enzyme mixture was kept up to date with a fork. This was required since utilizing the stirrer initially caused the beef to clump together (as depicted in Figure 3.6), impeding the stirrer's operation. It's important to Observe that after just one minute, adding the enzyme slurry, the stirrer resumed normal functionality. The hydrolysis process proceeded with continuous stirring (200 rpm) for the specified hydrolysis duration.

Upon completion of the hydrolysis process, the enzyme reaction was halted by transferring the beaker containing the hydrolysate to a shaking water bath (Grant Instruments, GLS 400, UK) set at $95 \pm 1^\circ\text{C}$ for 10 minutes, with intermittent stirring using a fork. Previous preliminary trials determined that enzyme activity ceased completely at 95°C . The slurry typically reached 95°C within approximately 5 minutes. Subsequently, the hydrolysate was cooled in an ice-water bath ($0 \pm 2^\circ\text{C}$) until it reached $18 \pm 2^\circ\text{C}$. It was then packaged into a plastic container, frozen, and stored at $-20 \pm 2^\circ\text{C}$ until further analysis.

Each hydrolysis reaction was conducted in duplicate using separate meat samples. In experiments exploring the impact of substrate concentration and pH on the hydrolysis distilled water was added to the procedure the beef meat samples as necessary to adjust the substrate concentration. Every duplicate specimen was examined at various stages of the hydrolysis reaction in triplicate.

General chemical analyses

Moisture content

The moisture content of both the meat and hydrolysates was assessed using the air oven drying method (Method 950.46, AOAC, 1991). Initially, two grams of minced meat or hydrolysate were meticulously weighed (Mettler Toledo, ML204, USA) to four decimal places and evenly spread into a pre-weighed moisture dish. Subsequently, the samples were dried at $105 \pm 1^\circ\text{C}$ in an air oven (Contherm, Digital series 2300, NZ) for 16 hours or until a steady weight was achieved.

After the drying process, once the samples had cooled to room temperature within a desiccator, the moisture dish containing the dried sample was re-weighed to the nearest ± 0.1 mg. Next, the moisture content was ascertained. by comparing the mass of the minced beef and meat hydrolysate before and after water evaporation in the oven.

$$\% \text{Moisture} = \frac{M_{\text{Initial}} - M_{\text{Dried}}}{M_{\text{Initial}}} \times 100$$

Where:

M_{initial} = initial weight of the sample.

M_{Dried} = weight from the specimen material after drying to constant weight.

Each determination of The amount of moisture was conducted in triplicate. Additionally, the total solids in minced beef and hydrolysates were calculated according to the quantity of solids remaining after evaporation of all water.

$$\% \text{ Total solids} = 100 - \% \text{Moisture}$$

Ash Content

The ash content of both minced beef and meat hydrolysates was assessed through dry ashing following air oven drying (Method 920.153, AOAC, 1991). Initially, ten grams of minced beef meat or meat hydrolysate were accurately weighed (Mettler Toledo, ML204, USA) into pre-weighed dried porcelain crucibles. After that, The samples were desiccated in an air oven (Contherm, Digital series 2300, NZ) at $105 \pm 1^\circ\text{C}$ for 12 hours to eliminate excess water.

Subsequently, the porcelain crucible containing the sample was heated over an open gas flame until the sample charred without emitting any smoke due to burning. Following this, the charred sample was placed in a muffle furnace (Vulcan®, A550, USA) capable of maintaining a temperature of $550 \pm 5^\circ\text{C}$ for 16 hours, until a white-grey ash was formed. Once it has cooled to ambient temperature, the porcelain crucible containing the ash was re-weighed (Mettler Toledo, ML204, USA) to the nearest ± 0.1 mg.

Each determination of ash content was conducted in triplicate. The calculation for ash content was derived by comparing the bulk of the minced meat or meat hydrolysate before and after the ashing procedure.

$$\text{Ash, \%} = \frac{\text{Weight of ash}}{\text{Weight of Sample}} \times 100$$

Fat Content:

The fat content in minced beef and meat hydrolysate was determined using the Mojonnier method (Mills et al., 1983). Initially, a meat emulsion was prepared by accurately weighing a 20 g sample of thawed minced beef or meat hydrolysate into a 200 ml beaker. To this emulsion, 100.00 g of 0.1 M NaOH was poured in, and the mixture was heated to a temperature of $70 \pm 2^\circ\text{C}$ for 5 minutes with occasional stirring.

The sample was immediately homogenized using a homogenizer (Success, Dyna Pasion, Malaysia) at high speed (20000 rpm) for four minutes. Afterwards, the homogenizer was rinsed with 50 ml pre-warmed distilled water to get rid of any meat residue, and the mass of the meat emulsion was adjusted to 200.00 g using distilled water.

Ten grams of the emulsion (equivalent to 1.00 g of minced beef or meat hydrolysate) was then weighed into a Mojonnier tube for solvent extraction. The extraction process consisted of two steps:

1. In the first extraction, a mixture of 1.5 ml ammonia, 10 ml ethanol, 25 ml ethyl ether, and 25 ml petroleum ether was used.
2. In the second step, extraction was done with the help of a mixture of 5 ml ethanol and 25 ml petroleum ether.

After adding the solvent mixture (extraction 1 or 2), the Mojonnier tube was manually inverted for about one minute to ensure thorough mixing. Subsequently, the samples were centrifuged (Funke Gerber, Super Vario-N, Germany) for two minutes. The upper layer containing the solvent with Carefully, fat was added to a pre-weighed beaker, leaving the meat or hydrolysate emulsion in the bottom part of the tube.

The beaker containing the solvent and fat was then heated on a hot plate (Stuart, CB302, USA) at minimum heat (approximately 40°C) inside the fume hood. This allowed the solvent to evaporate, leaving behind the fat within the beaker. The A second extraction was carried out utilizing the same procedure with fresh solvent. After centrifugation, the upper layer was included in the previous beaker, and The emulsifier was evaporated.

pH measurement

pH measurement of minced beef and meat hydrolysate samples

The acidity level of the minced beef and meat Samples of hydrolysate were quantified using the method recommended by Bendall (1973). First, a 0.5 to 1.0 g sample was added to approximately 10.0 ml of iodoacetate-KCl solution. Next, the blend was homogenized for 30 seconds using a handheld homogenizer.

Subsequently, the pH of the homogenate was measured at room temperature (25°C) using a standard pH meter equipped with a pb20 probe. Duplicate determinations were completed on each sample to ensure accuracy and consistency of the pH measurements.

pH measurement during meat hydrolysis process

During the hydrolysis reactions, the pH and temperature profile of the hydrolysates were monitored every 15 minutes. This was accomplished using a handheld pH meter equipped with a spear electrode that was placed inside the center of the reaction mixture.

After each pH measurement, distilled water was used to completely rinse the pH meter. to prevent any cross-contamination between samples. Additionally, Every day, the pH meter was calibrated using pH 7.0, pH 4, and pH 10 buffers to ensure accurate readings before the experiments commenced.



Figure 3.6: pH Meter

pH is shortened of “pond us hydro genii” It was put forth in 1909 to describe the extremely low concentrations of hydrogen ions by the Danish physicist S.P.L. Sorensen.

In 1909, pH was characterized as the inverse base 10 logarithm of the concentration of hydrogen ions. But the term was quickly modified because the hydrogen ion activity controls the majority of chemical and biological activities. Actually, ion activity was measured as a consequence of the initial potentiometric techniques. The definition that is now in use is the one based on hydrogen ion activity:

$$\text{pH} = -\log_{10}[\text{H}^+]$$

Table 3.1: reparation of a buffer mix

S.NO	Ph	PREPARATION
1	1.68	Dissolve 2.52gm of potassium tetra oxide in water to make the volume 200ml.
2	4.01	Dissolve 2.02gm of potassium bis phthalate dried at 110 degree centigrade for 1 hours in water to make the volume 200ml.
3	6.86	Dissolve 0.71 gm of dibasic sodium monobasic phosphate in water to make 200ml.
4	9.18	Dissolve of sodium tetra borate in water to make 200ml.
5	12.45	Saturated solution of calcium hydroxide in water at 25 0 C.

The buffer solution is then transferred to the glass bottle and label was pasted.

Standardization Procedure of pH Meter:-Select two buffer solution of standardization, whose difference in pH does not exceed four unit and such that the expected pH of the material under the falls between them. Fill the cell with Among the buffer solutions for standardization at the temp 25+2C at which, the test material is to be measured. Set the temp control at the temp of the solution and adjust the calibration control to make the observed pH value identical with the expected value. Rinse the electrodes and cell with it, at the same temp as the material to be measure. The pH of the second buffer solution is with +0.072. pH unit of the expected value. If large deviation is noted, examine the electrode if it is faulty replace it. Adjust the slope on temp control to make the observed pH value within expected value. Repeat the standardization, unit pH value reaches to 0.02pH unit of the expected value without further adjusting the control. After calibration, wash the

electrode with plenty of HPCL grade water to remove traces of any buffer. Take the solution of a sample in a beaker. After maintaining the temp of the beaker up to 25 C+2C and dip the electrode into the test solution. Now note the pH reading becomes steady. All solution to be examined and the reference buffer solution must be prepared using carbon dioxide free water.

Frequency of calibration:

The pH meter will be calibrated before every measurement.

In case if electrode gets break, new electrode will be activated by dipping in 0.1N HCL for pH. Calibration again.

Operation Procedure:

Switch on the power.

Keep on the instrument for 30 minutes before use. The determination is carried out the temp of 25 C unless otherwise specified in the individual monography

Select the mode by pressing “mode key”. The display will show “pH”.

pH measurement:

Remove the electrode from the purified water. Rinse with purified water and wipe out with tissue paper. calibrating the pH meter in accordance with calibration procedure.

Bring the temp of measuring solution to 25+or- 0 C and immerse the electrode in the solution.

Press “measured” key. The display will show pH value. Nolte the displayed value after “RDY” display appears.

After noting down the pH value of test solution, remove the electrode from solution. Wash with purified water and dip in purified water and press the “STAND BY”.

Total nitrogen content

The protein Material in the hydrolysates was established utilizing the Kjeldahl method with some modifications. Initially, sample sizes ranging from 0.8 to 1.0 g of minced beef and meat hydrolysate were accurately weighed and added to a 250 ml digester tube. Two Kjeldahl catalyst tablets were then included in the tube, then the inclusion of 20 ml of concentrated sulfuric acid.

The concoction was subjected to digestion for 45 minutes at 200°C, then heated to 270°C and held for 45 minutes. Subsequently, it was further heated to 330°C and held for 30 minutes, before finally reaching a temperature of 400°C and being digested until the sample became a clear solution, typically lasting around 3.5 hours.

After digestion the cooled substance was mixed with 70 ml of distilled water. Then, 70 ml of 40% NaOH was added to the tube, and was then positioned into a distillation unit. The resolution was distilled using direct steam injection, and the condensate from the distillation was collected in 50 ml of 4% w/w boric acid solution.

The boric acid solution containing the distillate was titrated with 0.1 M HCl until a grey-mauve color endpoint was reached. This titration process allowed in order to ascertain the protein content in the samples.

Non-protein nitrogen (NPN) content

Non-protein nitrogen (NPN) was determined in minced beef and meat hydrolysate to act as a sign of the extent of the hydrolysis reaction. Initially, protein was precipitated by adding 20 ml of 15% w/v trichloroacetic acid (TCA) to 10.0 ± 0.01 g of minced beef or meat hydrolysate.

The mixture was homogenized using a handheld homogenizer for one minute and then allowed to stand at ambient temperature for 30 to 45 minutes. Afterward Whatman No. was used to filter the homogenate. 1 filter paper, and the nitrogen content in the clear supernatant, which contained the NPN fraction, was determined.

For the NPN analysis, 25 ml of the clear supernatant was added to each 250 ml block digester tube, along with two Kjeldahl catalyst tablets. Exactly 18 After adding milliliters of concentrated sulfuric acid, the mixture was heated. for 60 minutes at 200°C, followed by heating at 270°C for 45 minutes, then at 330°C for 30 minutes. Finally, digestion was carried out at 400°C until the sample became a clear solution, typically lasting approximately 3.0 hours.

After digestion, the samples were diluted with 70 ml of distilled water. Changes were made to the method to prevent sample overflow from the tube in the digestion block due to the initially high water content.

Protein determination by Bradford method

The method employed to determine protein concentrations in hydrolysate samples and the enzyme slurry before SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis included applying the Bradford assay. Under acidic conditions, the Bradford reagent, which contains G250 Brilliant Blue stain, selectively binds to arginine and phenylalanine residues in protein molecules causing the creation of a blue-colored complex that can be quantified at 595 nm (Bradford, 1976).

To perform the assay, 5 ml of Bradford reagent was included in the sample solution, thoroughly mixed, and allowed to develop color for five minutes at room temperature. Subsequently, the absorbance of the final mixture was measured with a UV-Vis spectrophotometer at 595 nm. All determinations were conducted in duplicate to ensure accuracy and consistency of results.

The effect of reaction parameters on minced beef hydrolysis

A set of experiments was undertaken to look into how various reaction circumstances impact the levels of non-protein nitrogen (NPN) and total nitrogen in beef hydrolysates. The objective was to assess the impact of various factors such as reaction time, pH, temperature, additionally enzyme and substrate concentrations. These requirements were chosen in light of initial trials conducted by Vital Foods Ltd (unpublished data), aiming to ascertain the ideal parameters for reaching the intended outcomes in the hydrolysis process.

pH's impact on the extent of hydrolysis

based on the meat hydrolysis protocol outlined in Section 3.4.1, 80 grams of shredded beef were combined with 320 milliliters of reverse osmosis water. Dilution of the It involved minced meat. to facilitate adjustments Pertaining to the pH of the reaction solution and prevent gelatinization of undiluted meat, which can occur at low pH levels. Just before placing the mixture in the water bath, the minced beef and Distilled water was mixed together for one minute using a Waring blender (Waring, model HGBTWTS3 8010es, USA). The initial pH of the sample was measured using a Sartorius pH meter (model pb 20, Germany), and the pH was then adjusted to 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, and 8.0 ± 0.2 using 1 M HCl or 1 M NaOH, respectively.

Effect of substrate concentration on the extent of hydrolysis

In this experiment, the substrate concentration (beef meat) was adjusted by adding distilled water to the minced beef. The volume of purified water that was added to the beef was calculated using Equations 3.9 – 3.11 (Adler Nilsen, 1976). The enzyme concentration and processing temperature were kept constant at 2% w/w and $60 \pm 2^\circ\text{C}$, respectively. Each batch of the reaction mixture had a total mass of 240 grams, consisting of minced beef, water, and Zyactinase™. The percentage of protein was determined in the mixture of reactions using the initial protein content in the minced lean beef (PR%), which was measured to be 22.20 ± 0.06 grams of protein per 100 grams of meat.

$$MP = M \left(\frac{S\%}{100} \right)$$

$$MR = 1.1MP \left(\frac{100}{PR\%} \right)$$

$$MW = 1.1(M - M_{enz}) - MR$$

Where,

MP = Mass of utilized protein in grams

M = Total mass of the reaction mixture, including minced beef, distilled water, and enzyme, measured in grams.

S = The percent protein in the reaction mixture.

MR = The mass of minced beef in grams.

PR = The percent protein in minced lean beef.

MW = The mass of distilled water in grams.

M_{enz} = The mass of the enzyme solution.

The concentrations of substrates were adjusted from 2% protein up to $22.20 \pm 0.06\%$ w/w. At the maximum substrate concentration, there was no addition of distilled water. The substrate preparation, containing water and meat only, was homogenized for one minute at medium speed in a Waring blender to ensure a homogeneous composition before it was placed in the water bath.

Temperature's impact on the degree of hydrolysis

In this trial, we started with a minimum temperature of 40°C , without adjusting the pH. However, we monitored pH levels initially and throughout the process. Following the guidelines from Yamaguchi et al. (1982), we chose temperature ranges of 40°C , 50°C , 60°C , 65°C , 70°C , and 75°C , all maintained within a tolerance of $\pm 2^{\circ}\text{C}$. Before adding the enzyme slurry, The ground beef was cooked to the specified temperature as per the procedure outlined in Section 3.4.1. After varying hydrolysis durations from 1 to 180 minutes, the resulting hydrolysate was cooled to $18 \pm 5^{\circ}\text{C}$ in an ice water bath and then stored at $-20 \pm 2^{\circ}\text{C}$ after packaging and freezing for subsequent analysis.

Enzyme concentration's impact on the degree of hydrolysis

This study aimed to investigate how varying concentrations of ZyactinaseTM affect The magnitude of hydrolysis. The amount of different enzyme concentrations applied to the hydrolysis mixture according to the initial weight of raw minced beef. The enzyme concentrations tested were 1%, 2%, and 4% w/w. For instance, when using a 2% enzyme concentration, $2.00 \pm 0.01\text{g}$ of An enzyme was combined with 100 g of meat in a slurry with 8 ml of warm reverse osmosis water just before incorporation into the minced beef.

Polyacrylamide gel electrophoresis (SDS- PAGE) analysis

Gel preparation

SDS-PAGE was conducted following the protocol outlined by Laemmli (1970) using the Bio-Rad Mini-PROTEAN® Tetra Cell multi-casting chamber (Bio-Rad Laboratories Inc., USA) to fabricate gels with a thickness of 1.5 mm. Gel preparation followed the guidelines provided in the Bio-Rad Mini Protean(R) 3 cell instruction manual, detailed in Table 3.1. The separating gel was filled to a height of about 2 cm from the top plate before the gel mixtures were carefully poured into the casting modules. Distilled water was gently layered on top of the separating gel to guarantee a level surface and shield it from oxygen. After polymerization, which takes 45 to 60 minutes, the distilled water was replaced with a 4% stacking gel, filling up to the top of the short plate. Next, the comb was placed at the top of the short plate to form wells. Once the stacking gel had polymerized (30 to 40 minutes), the comb was removed, and the wells were rinsed with distilled water and running buffer to remove any trapped air. The gel should be used within 24 hours of preparation.

Sample preparation for SDS-PAGE

The samples were diluted to achieve a final protein concentration of 2 mg/ml in a sample buffer. Following dilution, the samples were thoroughly mixed with a vortex mixer for one minute, then heated at 95°C for five minutes, and subsequently samples were centrifuged at $6,000 \times g$ for five minutes. Broad-range molecular weight markers were used to estimate the relative molecular weights of the proteins.

Staining and destaining for SDS-PAGE

Right after completing the electrophoresis, the gels were removed from the plates and immersed in Coomassie Brilliant Blue R-250 solution obtained from Bio-Rad (Bio-Rad Laboratories Inc., USA) for a duration of 30 to 45 minutes. Subsequently, the gels underwent rinsing with distilled water and were left to soak in destaining solution overnight.

SDS-PAGE imaging

The gel pictures were taken using a scanner (Canon, CanoScan LiDE 20, USA), and subsequent analysis involved comparing the measured molecular weights in the SDS-PAGE pattern with those of the Precision Plus Protein™ Dual Colour Standards norms for molecular weight acquired from Bio-Rad Laboratories Inc.

Peptide analysis on hydrolysates

Sample preparation

The hydrolysate sample was thinned with 0.1% trifluoroacetic acid (TFA) to achieve a 1:5 dilution ratio. The sample underwent homogenization for 8 minutes using a stomacher and was later centrifuged at 10,000 x g for 20 minutes using a high-speed centrifuge (IECB-22M, USA). After then, the supernatant was moved into a 5 ml plastic syringe and filtered through a series of 0.45 µm and 0.2 µm before being injected into the syringe, filters high-performance liquid chromatograph (HPLC).

Conditions for chromatography in peptide analysis

The HPLC system employed for the separations included a Shimadzu SCL10AVP pump with LC 10ATV system controller and Diode array detector SPD M10AVP (Shimadzu, Japan). A 10 µl portion of the filtrate from Section 3.9.1 was introduced into the HPLC. A was used to separate the peptides using a 250 x 4.6 mm Jupiter 5µ C18 300A column (Phenomenex, USA) maintained at 40 ± 1°C. The mobile phase comprised solvent A containing 0.1% TFA (optima LC/MS, Fisher Scientific, UK) in distilled water, and solvent B containing 60% acetonitrile and 0.085% TFA (optima LC/MS, Fisher Scientific, UK) in distilled water. Solvent C, containing 60% acetonitrile, was utilized to rinse the HPLC system post each run. Prior to each analytical run, all mobile phases (A, B, and C) were passed through a 0.20 µm nylon filter (Millipore, USA) and degassed. The HPLC gradient commenced with an initial isocratic gradient of 1% solvent B for 5 minutes, followed by a linear gradient from 1% to 60% solvent B over 20 minutes. At the conclusion of each run and before the subsequent injection, the system was purged with 60% acetonitrile (solvent C) for 5 minutes. The separation was monitored at a UV wavelength of 214 nm.



Figure 3.7: Superior Liquid Chromatography Performance

INTRODUCTION: High-performance liquid chromatography (HPLC), also known as high-pressure liquid chromatography, is a chromatographic method that can be used to identify, quantify, and purify each of the elements of an amalgam by separating it into its constituent parts. It is an effective analytical tool. It is a type of liquid chromatography that makes use of higher fluid phase pressures, smaller media inside the column, and smaller column sizes.

A mobile phase reservoir, a pump, an injector, a separation column, and a detector make up an HPLC device. By injecting a plug of sample mixture onto the column, compounds are separated. Due to variations in how they partition between the stationary phase and the mobile liquid phase, the various components of the mixture travel through the column at varying rates. Following its passage down the column, the mobile phase enters the detector, which tracks the various molecules as they move through it and gives the analyses a distinctive retention period. The detector sends a signal to a printer, which displays the separation graphically.

TYPES OF HPLC TECHNIQUES:

1. According to chromatographic modes

- Chromatography in the normal phase
- Phase-reversal chromatography

2. Based on the separation concept

- Adsorption chromatography
- Chromatography using Ion Exchange
- Ion pair chromatography
- Chromatography using size exclusion
- Affinity chromatography
- Chromatographic separation of phases

3. According to the elution method

- Isocratic separation
- Gradient separation

4. Based on the scale of operation

- Analytical HPLC
- Preparative HPLC

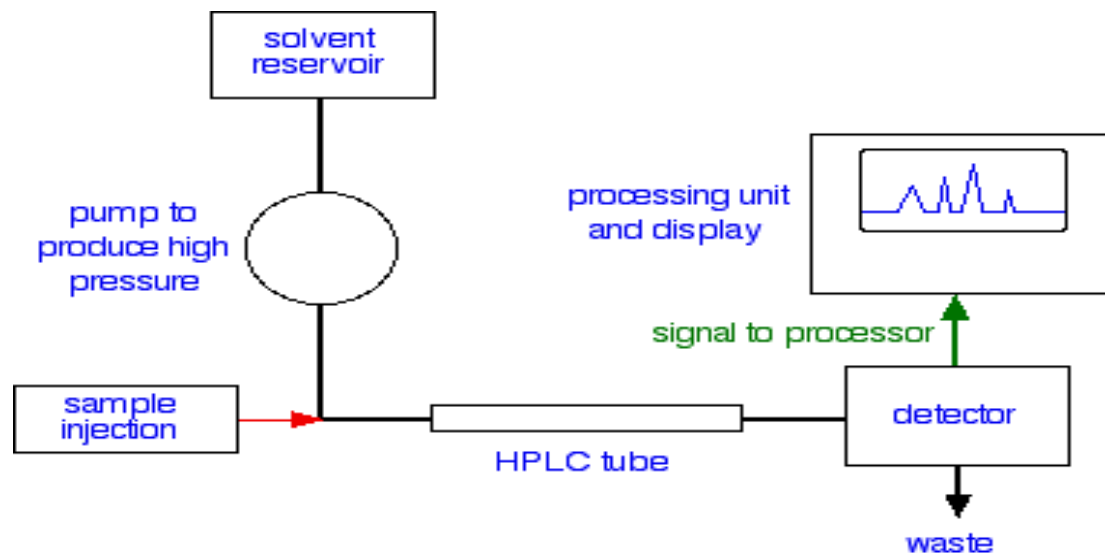
INSTRUMENTATION:

Figure 3.8: An HPLC flow chart

To prevent bubble formation, solvents must be degassed. The pumps in the system provide a consistent high pressure without pulsation and can be programmed to alter the solvent composition throughout the separation process. A liquid sample is introduced into the sample loop of an injector using a syringe. Once the loop is filled, the injector can insert the sample into the mobile phase stream by aligning the sample loop with the mobile phase tubing. Details about the various types of HPLC columns are covered in a separate document. The presence of analytes in the column effluent is detected by monitoring changes in refractive index, UV-Vis absorption at a specific wavelength, fluorescence after excitation with an appropriate wavelength, or electrochemical response.

The key elements of an HPLC system include:

- Solvent Reservoir
- Pump
- Injection Port
- Column
- Detector
- Data Acquisition System

Solvent Reservoir: The Solvent Reservoir is used to hold the mobile phase. Scott Duran bottles are commonly utilized for this purpose. The reservoir must be constructed from inert materials like glass and have a smooth surface to prevent microbial growth. It can be either transparent or amber-colored. A graduated bottle allows for a rough estimation of the mobile phase volume. Solvent reservoirs are typically positioned on a tray above the HPLC system, but they should not be placed directly above the system to avoid potential damage from any solvent spillage onto the electronic components.

HPLC Pump: The HPLC Pump is a vital part of the system, providing a continuous flow of the mobile phase or phases to facilitate the separation of mixture components in an efficient manner. It operates in one of two modes: Isocratic or Gradient.

Modes of HPLC:

Isocratic: In isocratic elution, compounds are separated using a constant mobile phase composition throughout the entire run. The mobile phase composition remains unchanged during the process, ensuring that all conditions are kept constant.

Slope: A deliberate change is introduced during the specific process

A deliberate change is incorporated during the sample run to achieve better and/or faster separation. In gradient elution, the composition of the mobile phase is continuously adjusted throughout the run to enhance the separation process. There are two types of gradient systems used for this purpose:

- Mixing at low pressure
- High pressure mixing

Each system offers its own advantages. Mixing at low pressure provides the option to use three or four different mobile phases. In high-pressure mixing, typically two pumps are employed, which limits the choice to only two different mobile phases. One advantage of high-pressure mixing is that, even if one pump fails, you can continue to operate in isocratic mode..

Injection port: The sample introduction device, such as an injector, is designed to introduce the sample into the high-pressure flow of the mobile phase. Unlike gas chromatography (GC), where direct syringe injection onto the column is possible, liquid chromatography (LC) requires a different approach due to the high inlet pressure. A widely used method is valve injection, which utilizes a fixed or variable loop, with the Rheodyne valve being the most common device. These loops can be filled either partially or fully, with injectors available for both options. The advantage of partial loop filling is that it allows the use of a smaller sample volume, which is beneficial when the sample is limited.

HPLC Column: The HPLC column contains the stationary phase, which is essential for separating the components of the sample. These columns are typically constructed from SS-316 grade stainless steel. In addition to the columns, the materials used for tubing, fittings, plumbing, and connections are also crucial to the system's performance.

HPLC Detectors: Detectors identify various compounds as they elute from the column, providing a response in the form of a millivolt signal. This signal is then processed by the computer (or integrator) to generate a chromatogram. Essentially, the detector consists of a flow cell through which the mobile phase and the separated sample components pass.

In pharmaceutical analysis, Ultraviolet/Visible (UV/Vis) absorption spectrometers are among the most commonly utilized detectors. A UV detector functions by measuring the absorbance of UV light by chromophores within the analyte compound. Meanwhile, a refractive index detector detects changes in the refractive index of the mobile phase as the analyte-enriched stream flows through the cell. Similarly, a fluorescence detector is used to monitor the fluorescence emitted by specific compounds.

The Photo Diode Array (PDA) Detector is currently the most widely used detector in liquid chromatography (LC).

Different Types of HPLC Detectors

- Universal detector – Refractive Index Detector
- UV-Visible Detector
- Fluorescence Detector

Data Acquisition System: The data system processes the detector output and integrates it to create a meaningful chromatogram. Modern integration systems go beyond this, offering features such as chromatogram processing, calculations, statistical analysis, and data backup and storage. Additionally, data systems can control various parameters of the HPLC system.

System Suitability: The system suitability test is a crucial component of the method, used to verify the proper functioning of the chromatographic system. It evaluates the performance of the chromatography system and provides a graphical summary of its effectiveness. Key parameters used to assess column performance include efficiency, capacity factor, resolution factor, and symmetry factor. These terms are defined as follows. Factors

that can affect chromatographic behaviour include mobile phase composition, temperature, ionic strength and apparent pH, flow rate, and column length, and stationary phase Attributes like porosity, particle size and type, specific surface area.

PARAMETERS USED IN HPLC:

1. Retention time (Rt):

The duration required for a specific compound to move through the column and reach the detector is referred to as its retention time. This duration is recorded from the moment the sample is injected until the display registers the maximum peak height for that compound. Retention times vary across different compounds. For any given compound, the retention time can fluctuate based on:

- The applied pressure (as it influences the solvent flow rate)
- The characteristics of the stationary phase (including its material and particle size)
- The precise composition of the solvent
- The column's temperature

Therefore, careful control of conditions is essential if retention times are to be used for identifying compounds. Retention time is the required for The elution of 50% of a component from the column. Retention time is recorded in either minutes or seconds.

2. Volume of Elution (Vr): This term refers to the volume of mobile phase required to elute 50% of a component from the column and is determined by multiplying the retention time by the flow rate.

To find the retention volume, multiply the retention time by the flow rate.

3. Resolution in HPLC Resolution (Rs): The concept of resolution refers to the extent of separation between two components and how well they are separated from the baseline. It is usually defined as the difference between the centers of two eluting peaks, measured in retention time or volume, divided by the average width of the peaks. For instance, an Rs value of 1.0 indicates that 98% purity has been achieved, assuming a peak recovery rate of 98%. Full baseline separation between two clearly defined peaks reflects 100% purity and requires an Rs value greater than 1. The resolution between two peaks of similar height in a chromatogram can be determined using the following formula:

USP Resolution:

$$R_s = 2.0 \frac{Rt_2 - Rt_1}{W_2 - W_1}$$

Where,

R_s = Resolution

Rt_1 = Time recorded for the first peak

Rt_2 = Time recorded for the second peak

W_1 and W_2 = Total width of peaks at the baseline, measured where lines tangent to the peaks at 50% height intersect

EP and JP Resolution:

$$R_s = 1.18 \frac{Rt_2 - Rt_1}{W_2 - W_1}$$

4. Efficiency (N): The productivity of a chromatographic column is Described in terms of the theoretical plates (N) and can be computed with the following formula:

USP Plate Count:

$$N = 16 [Rt/W]^2$$

Where,

N = Quantity of theoretical plates

Rt = Dwell time

W = Apex width at baseline determined by tangents drawn to 61% of peak height

EP Plate Count: $N = 5.54 [Rt/W_{1/2}]^2$

Where, $W_{1/2}$ = Apex width at 50% of peak height

JP Plate Count $N = 5.55 [Rt/W_{1/2}]^2$

Where, $W_{1/2}$ = Apex width at 50% of crest elevation

Various factors lead to an increase in the column plate number:

- Well Filled columns (column 'standard')
- Longer columns
- Decreased flow rates (without going too low)
- Minimized column packing particles
- Less viscous mobile phase and raised temperature
- Smaller sample molecules

As the number of theoretical plates in a column increases, so does its efficiency and correspondingly The more precise the resolution that can be obtained through column length.

5. Retention Coefficient (K') : The capacity factor k' is tied to the retention time, representing the fraction of time a solute remains in the stationary phase rather than the mobile phase. Higher values of k' result from longer retention times of K'. It measures the dwell time of a sample molecule relative to the analytical column dead volume. For each peak in a chromatogram, the capacity factor K' can be calculated with the following equation:

$$K' = \frac{Rt - V_o}{V_o}$$

Where,

K' = Capacity factor

Rt = Retention time

V_o = Void volume time

6. Selectivity Factor (a): Selectivity (a) Is comparable to the relative retention of the solute peaks, whereas efficiency is highly influenced by the chemical characteristics of the chromatographic medium. It measures the Comparative retention of two peaks in a chromatographic profile (the ratio of two K' values). Selectivity is never measured for the first peak in a chromatographic profile because there is no preceding peak to use in the calculation.

Selectivity for peak at Rt_2 :

$$a = \frac{Rt_2 - V_0}{Rt_1 - V_0}$$

Where,

a = Selectivity

Rt_1 = Dwell time of the first peak

Rt_2 = Dwell time of the second peak

V_0 = Void volume time

7. Tailing or Symmetry Factor (T): It calculates the asymmetry of a peak. For pharmaceutical purposes, the Peak asymmetry (T) is Explained as the range separating the leading edge and tailing Margin of the crest at a width of 5% of 14 the peak the leading height divided by twice the distance (F) between the Apex of the peak and the Front margin of the summit at 5% of peak height. It is given by the equation as follows:

$$T = \frac{W}{2F}$$

Where,

T = Tailing Factor

W = Apex width at 5 % of peak height

F = Time from width start at 5 % of peak height to retention time

Table 3.2: The chromatographic gradient programme used for peptides separation

Time C (Minutes)	Flow Type	Solvent A	Solvent B	Solvent C
		0.1 % v/v TFA	60% acetonitrile and 0.085% TFA in water	60%
0 – 5	Isocratic	99% Solvent A	1% Solvent B	-
5 – 20	Gradient	90% to 40% Solvent A	1% to 60% Solvent B	-
20 – 25	Isocratic	-	-	100%

Chromatograph analysis

The evaluation of chromatographic conditions was conducted by injecting a synthetic peptide standard mixture (Sigma Aldrich, H2016-1VL, USA) with peptides of varying molecular weights into the column. The peptide mixture consisted of 0.5 mg each of five distinct protein peptides: (1) GLY-TYR, with a molecular weight of 238.2 g/mol, (2) Methionine Enkephalin Acetate, MW = 573.7 g/mol for free base (TYR-GLY-GLY-PHE-MET), (3) VAL-TYR-VAL, MW = 379.5 g/mol, (4) Leucine Enkephalin, MW = 555.6 g/mol for free base (TYR-GLY-GLY-PHE-LEU), and (5) Angiotensin II Acetate, MW = 1046.2 g/mol for free base (ASP-ARG-VAL-TYR-ILE-HIS-PRO-PHE). The chromatographic profiles of the samples were compared before and after hydrolysis to evaluate the degradation of proteins throughout the hydrolysis process. All analyses were conducted in duplicate.

Amino acid analysis on hydrolysate

Sample preparation

Samples of minced meat and hydrolysates were prepared for individual amino acid analysis by precipitating proteins with 5% TCA, following the procedure outlined by Mullen et al. (2000). Initially, minced meat and hydrolysate samples were blended with MilliQ reverse osmosis water (dilution ratio 1:2) in a stomacher (IUL instruments, Basic, Spain) for one minute. Subsequently, 50% TCA was added to achieve a final concentration of 5% TCA in the solution. The mixture was then cold-centrifuged ($4 \pm 1^\circ\text{C}$) at $3600 \times g$ for 20 minutes using an IECB-22M centrifuge (USA). The resulting supernatant was passed through Whatman No. 1 filter paper,

and a 250 μL aliquot was transferred to a 2 mL microcentrifuge tube. Following this, 250 μL of each sample, along with 50 μL of an internal standard solution (5 mM neoleucine), were vortex-mixed for one minute. Finally, 20 μL of the supernatant was derivatized to their phenylthiocarbonyl derivatives based on the method described by Bidlingmeyer et al. (1987).

Sample derivatization for amino acids

The supernatant, containing either the free amino acid standard or the hydrolyzed sample, was transferred into a 2 mL centrifuge tube and subjected to drying using the Speed vac (Eppendorf, Germany) at 30°C for 10 minutes. After drying, a reagent mixture composed of methanol, distilled water, and triethylamine in a 2:2:1 ratio was added to rehydrate the resulting pellet. Following another round of drying with the Speed vac, the sample was prepared for derivatization.

To achieve the formation of PITC amino acids, the dried samples were subjected to treatment with 20 μL of a derivatization reagent and permitted to react for 20 minutes at ambient temperature. Following this, surplus reagents were eliminated under vacuum using the Speed vac. Upon completion of the derivatization process, the dried sample was reconstituted with the dried sample was rehydrated with 400 μL of diluent containing 5 mM sodium phosphate (pH 7.6) with 5% v/v acetonitrile. The prepared sample was subsequently then ready for separation using HPLC.

Chromatographic parameters for amino acid evaluation

The amino acids, once derivatized, underwent separation using a Shimadzu HPLC system, which included an SCL-10AVP pump with LC 10ATV system controller and SPD M10AVP diode-array detector (Shimadzu, Japan). Injecting a 10 μL portion of the derivatized amino acid sample into a Gemini® 5 μm C18 110 Å LC 30 x 2 mm column maintained at $40 \pm 1^\circ\text{C}$ facilitated the separation. Utilizing two mobile phase solvents at a rate of 0.8 mL/min, with solvent A comprising a 0.14 M sodium acetate trihydrate solution with 0.5 mL of TEA (HPLC grade, Fisher Scientific, UK), adjusted to pH 6.40 using acetic acid, and solvent B consisting of acetonitrile:water (60:40 v/v), ensured effective separation. The chromatographic gradient program is outlined in Table, and monitoring occurred at a UV wavelength of 254 nm.

Table 3.3: The chromatographic gradient protocol employed for amino acid separation

Time (minute)	Flow Type	Solvent A(%)	Solvent B(%)
0	Isocratic	90.0	10.0
0 – 6	Gradient	90.0 – 87.5	10 – 12.5
6 – 38	Gradient	87.5 – 42.0	12.5 – 58.0
38 – 71	Gradient	42.0 – 0	58 - 100
71 – 79	Isocratic	0	100
79 – 81	Gradient	0 – 90.0	100 - 10

Chromatograph analysis

A mixture of amino acid standards was created by combining 95 microliters of a 250 pmol/ μL amino acid calibration mixture (Sigma-Aldrich, USA) with 5 microliters of a 10 millimolar Norleucine. Following the same protocol outlined in Section 3.10.2, this mixture underwent derivatization. Additionally, individual 10 mM amino acids (Sigma-Aldrich, USA) were separately prepared and subjected to the same derivatization procedure as detailed in Section 3.10.2. The resulting chromatograms of the separate amino acids were subsequently compared to those of the amino acid standard mixture to ascertain the elution order of amino acids.

Kinetic studies of meat hydrolysis

Kinetic investigations into the hydrolysis process were conducted consistently at 60°C without any pH adjustments. Two distinct sets of experiments were undertaken:

1. In the first set, the enzyme concentration remained constant while the minced beef concentration varied.
2. In the second set, the minced beef concentration remained fixed while the enzyme concentration varied.

To achieve different substrate concentrations, purified water was incorporated into minced beef to formulate a 240 g reaction mixture containing minced beef, reverse osmosis water, and ZyactinaseTM enzyme. The addition of water to reach specific substrate concentrations.

To ensure a homogeneous substrate, minced beef and reverse osmosis water were blended using a Waring blender for 30 seconds at high speed followed by 30 seconds at low speed. Subsequently, the homogenized mixture was transferred to a 500 ml beaker before being Situated in the temperature-controlled water bath.

The reaction blend was heated to $60 \pm 1^\circ\text{C}$, and it took approximately 15 minutes for the mixture to attain the desired temperature. Enzyme concentrations of 2%, 4%, and 7.5% w/w, relative to the overall mass of the reaction mixture, were selected for the study.

Initially, samples of the hydrolysate were collected at one-minute intervals during the first five minutes of hydrolysis. Subsequently, samples were obtained at 15, 30, 45, 60, and 120-minute intervals.

OPA analysis for amino acids and degree of hydrolysis (DH)

he concentration of α -amino acids was determined utilizing the ortho-Phthalaldehyde (OPA) method as described by Nielsen (2001). Serine standards were prepared by dissolving serine in distilled water at concentrations ranging from 0.005 to 0.05 M. A reference standard was prepared by diluting 50 milligrams of serine with 500 ml of distilled water (0.9516 meq/l).

For the meat and hydrolysate samples, accurately weighed amounts ranging from 0.1000 to 1.0000 g were combined with purified water and made up to 100 ml in a volumetric flask. The assay procedure involved adding 400 μl of serine standard or diluted sample into 3 ml of OPA reagent, followed by mixing for five seconds. The mixture was left to stand for exactly two minutes before being read at 340 nm in a UV-Vis spectrophotometer.

The extent of hydrolysis (DH) represents the percentage of peptide bonds hydrolyzed during hydrolysis, calculated using hydrolysis equivalent (heq), which relates to the quantity of peptide bonds broken during hydrolysis, expressed as meq/g protein. Hence, $\text{DH} = (\text{heq}/\text{htotal}) \times 100$, is the sum of the millimoles of individual amino acids per gram in the unhydrolyzed protein.

Challenges in collagen extraction, ranging from denaturation to variability in sources, have been addressed by innovative approaches. M. J. Buehler and S. L. Mayo have investigated computational methods to predict and mitigate denaturation risks. Innovations in the 21st century, such as the development of synthetic collagens by J. R. Sanes and advanced extraction protocols, highlight the ongoing efforts to overcome challenges.

Denaturation and Loss of Biological Activity:

Challenge: Collagen is sensitive to denaturation, which can result in the loss of its native structure and biological function through the extraction process.

Impact: Denaturation can affect the functionality of the extracted proteins, reducing their effectiveness in applications such as tissue engineering or drug delivery.

Source Variability:

Challenge: Collagen is found in various tissues with different compositions and qualities. Variability in collagen sources can impact extraction efficiency as well as the attributes of the extracted proteins.

Impact: Inconsistencies in collagen source properties may lead to differences in the quality and characteristics of the extracted proteins, affecting their suitability for specific applications.

Immunogenicity:

Challenge: Extracted collagen may induce immune responses when introduced into biological systems, particularly in medical uses such as tissue regeneration or regenerative medicine.

Impact: Immunogenic responses can limit the effectiveness of extracted collagen in vivo and may lead to adverse reactions, necessitating strategies to reduce immunogenicity.

Environment Impact of Chemical Method:

Challenge: Traditional chemical extraction methods often involve the use of harsh solvents, raising environmental concerns.

Impact: The environmental impact of chemical methods, including the generation of hazardous waste, poses challenges regarding sustainability and eco-friendliness.

Yield and Purity Optimization:

Challenge: Achieving high yields of pure collagen proteins is a complex task and depends on various extraction parameters.

Impact: Lower yields or impurities in the extracted proteins can affect downstream applications, requiring optimization of extraction protocols for enhanced efficiency.

Standardization of Methods:

Challenge: There is an absence of standardized methods for collagen extraction, leading to variations in protocols across studies and laboratories.

Impact: Lack of standardization makes it challenging to evaluate findings across various studies and hinders the establishment of universally accepted protocols.

Eco-Friendly Practices:

Challenge: Balancing the need for high extraction yields with eco-friendly practices can be challenging, especially in chemical extraction methods.

Impact: The environmental impact of extraction processes is becoming increasingly important, and the advancement of sustainable and green extraction methods presents a challenge for the field.

Explore how variations in hydrolysis conditions impact both total nitrogen and non-protein nitrogen contents.

The enzymatic hydrolysis of beef using Zyactinase may be influenced by various factors such as pH, temperature, reaction time, and enzyme and substrate concentration. While kiwifruit extract or the enzyme has been thoroughly investigated for its effects on different proteins, the use of Zyactinase™, a patented enzyme complex, has not been completely explored in relation to its impact on beef hydrolysis. This chapter aims to investigate the properties of Zyactinase™ and optimize the reaction conditions for beef meat hydrolysis. The evaluation of Zyactinase™ hydrolysis of meat involves monitoring the release of non-protein nitrogen (NPN), indicating the breakdown of protein moieties into amino acids or smaller peptides with molecular weights less than 800 Daltons. Additionally, SDS-PAGE profiles of meat hydrolysates are analyzed to track changes in protein composition.

The Zyactinase™ enzyme complex

The Zyactinase™ is derived from kiwifruit and is a semi-purified freeze-dried extract. It comprises various components, including proteolytic enzymes, carbohydrates, fiber, and numerous micronutrients naturally present in the original fruit. The moisture content of Zyactinase™ is 2.5%. To analyze its protein composition, 1.0 g of Zyactinase™ powder is dissolved in 1.0 milliliters of purified water, and the protein content is assessed using Bradford analysis. Subsequently, the Zyactinase™ solution is diluted up to 1 mL with SDS-PAGE sample buffer to achieve an amount of 1.0 g protein per milliliter of sample buffer. The SDS-PAGE analysis reveals distinct bands around 28 kDa, 24 kDa, 23 kDa, and 17 kDa, with one less clear band around 27 kDa in the Zyactinase™ sample (Z). The SDS-PAGE pattern of Zyactinase™ is consistent with

patterns observed in raw kiwifruit extract as reported in previous studies (Afshar-Mohammadian, Rahimi-Koldeh, & Sajedi, 2011; Tuppo et al., 2008).

According to Larocca, Rossano, & Riccio (2010), green kiwifruit extract typically exhibits six protein bands within the 17 – 30 kDa range. Specifically, the band at 30 kDa is identified as actinidin, while the band at 26 kDa corresponds to kiwellin. Additionally, bands at 25 and 24 kDa are associated with thaumatin-like protein (TLP) (Larocca, Rossano & Riccio, 2010). Another band observed at 20 kDa is named KiTH (Tuppo et al., 2008), and the lower band at 17 kDa may correspond to a propeptide of actinidin (Pastorello et al., 1998).

Actinidin, identified as the major kiwifruit protease, contains a free sulfhydryl group Essential for its enzymatic function (McDowall, 1970) and is classified as a plant thiol protease. It consists of 220 amino acids with a molecular weight of 23 kDa (Kamphuis, Drenth, & Baker, 1985). While Carne & Moore (1978) suggested the presence of a single enzyme in *Actinidia chinensis* kiwifruit, Bolland & Hardman (1972) reported the identification of two active proteins with a molecular weight of 26 kDa through wide chromatography column analysis on DEAE cellulose.

Furthermore, Sugiyama et al. (1996) discovered six acidic isoforms of actinidin with slightly varying isoelectric points (pI) but identical N-terminal sequences. Through ion exchange chromatography, Sugiyama et al. (1997) purified two kiwifruit proteases, kp4 and kp6, demonstrating differing substrate specificities and pH optima for two of these acidic isoforms. Actinidin exhibits multiple isoforms with the same pI but varying molecular masses (Larocca, Rossano & Riccio, 2010).

Variations in pH levels throughout the hydrolysis process were closely monitored and recorded to assess their impact on the enzymatic breakdown of proteins

The hydrolysis process proceeded without alterations to the pH or the addition of water initially. pH levels were meticulously recorded before enzyme addition (0 minute), immediately after enzyme addition (one minute), and at 20-minute intervals throughout the 180-minute hydrolysis period. The findings, illustrated in Figure 4.4, indicate that the pH of the raw beef, processed without Zyactinase™ (control), remained unchanged at all stages of the process. Conversely, in samples supplemented with Zyactinase™, a notable decrease in pH was observed at the onset of hydrolysis, attributed to the inherent acidity of Zyactinase™. Although there was a slight increase in pH during hydrolysis, the final hydrolysate exhibited a lower pH compared to the initial minced meat.

The decline in pH is likely influenced by the acidic nature of the enzyme complex, given that Zyactinase™ has a pH of 3.1. Another contributing factor could be the liberation of carboxyl groups from the protein chain by the proteolytic enzyme. However, the substantial pH drop upon Zyactinase™ addition, prior to significant hydrolysis, suggests an alternative mechanism. It is plausible that this pH shift results from structural alterations in meat proteins induced by Zyactinase™, exposing amino acids previously sequestered within the protein's 3-D structure.

The subsequent stabilization of pH following the initial decrease may be attributed to the robust buffering capacity inherent to animal muscle. This buffering capacity is thought to be conferred by various constituents, including inorganic phosphate, nucleotides, organic acids, taurine, and histidine residues bound to proteins. Generally, meat muscle exhibits effective buffering within the pH range of 6.5 to 7.5, with red fibre muscle displaying lower buffering capacity than white muscle under acidic conditions.

CHAPTER – 4

CONCLUSION

$$\text{Protein Content (\%)} = \frac{(\text{Blank}) - (\text{B.R})}{0.1 \times 1.005} = \frac{45.8 - 34.6 \times 6.25 \times 0.999 \times 1.4 \times 100}{0.1 \times 1.005} = 97.85\%$$

BR = Burette Reading

0.999 = Actual Molarity NaOH

1.4 = Factor

0.1 = Standard Molarity NaOH

1.005 = Weight of Sample

Limit = NLT 95% (Sample which are employed for extraction process)

Collagen, as a crucial structural protein, offers great promise for a range of biomedical uses, spanning from tissue engineering to drug delivery. Throughout this thesis, we have explored innovative approaches to collagen extraction aimed at preserving its native structure and enhancing its bioactivity. By comparing traditional methods with enzymatic hydrolysis and supercritical fluid extraction (SFE), we have demonstrated the superiority of the latter in producing high-quality collagen with superior purity, yield, and bioactivity.

Our investigation into enzymatic hydrolysis revealed its ability to selectively cleave peptide bonds within collagen molecules, resulting in collagen products with minimal degradation and enhanced bioactivity. Similarly, supercritical fluid extraction (SFE) emerged as a promising alternative, offering a solvent-free and eco-friendly approach to resource extraction collagen with exceptional purity and stability.

The experimental Conclusions provided in this thesis underscore the importance of optimizing extraction protocols to maximize collagen yield upholding its structural soundness and functional properties. SDS-PAGE analysis, FTIR spectroscopy, and cell culture assays provided valuable insights into the composition, conformation, and bioactivity of extracted collagen, validating the efficacy of innovative extraction techniques.

Looking ahead, The results of this thesis hold considerable significance for biomedical research and clinical practice. Collagen-based biomaterials derived from enzymatic hydrolysis and SFE hold promise. The findings of this thesis have important implications for various fields, such as repairing injuries and regenerating tissues, and drug delivery systems. The customizable properties of extracted collagen offer exciting opportunities for tailoring biomaterials to specific therapeutic needs, paving the way for personalized medicine and precision healthcare.

However, challenges remain in scaling up production and translating laboratory findings into clinically viable solutions. Further research is needed to optimize extraction protocols, enhance collagen stability and functionality, and explore novel applications in emerging fields such as regenerative medicine and 3D bioprinting.

This thesis contributes to the growing body of knowledge on collagen extraction techniques, providing researchers with valuable information to harness the therapeutic potential of this remarkable protein.

CHAPTER – 5

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CHAPTER – 6

Appendix

Appendix A: Materials and Equipment

Materials:

- Bovine skin samples
- Hydrochloric acid (HCl)
- Pepsin enzyme
- Trypsin enzyme
- Collagenase enzyme
- Sodium chloride (NaCl)
- Ethanol
- CO₂ (for supercritical fluid extraction)
- Distilled water
- Sodium Hydroxide (NaOH)

- Copper Sulphate (CuSo4)
- Methyl Red Indicator

Equipment:

- Analytical balance
- pH meter
- Centrifuge
- Shaking incubator
- UV Spectrophotometer
- Sonicator
- High-pressure reactor (for supercritical fluid extraction)
- Freeze dryer
- Kjeldhal Flask

Appendix B: Experimental Procedures**Enzymatic Hydrolysis:**

Preparation of collagen source (bovine skin samples)

Acid pretreatment: Treatment of collagen source with hydrochloric acid (pH 2) for 24 hours.

Enzymatic digestion: Incubation of acid-treated collagen with pepsin/trypsin/collagenase enzyme at optimal conditions (temperature, pH) for 48 hours.

Filtration: Separation of digested collagen solution from undigested residues using a filter.

Dialysis: Dialysis of collagen mixture compared to distilled water to remove excess enzyme and salts.

Freeze drying: Freeze drying of dialyzed collagen solution to obtain collagen powder.

Supercritical Fluid Extraction

Preparation of collagen source (bovine skin samples)

Grinding: Grinding of collagen source into fine particles.

Loading: Loading of ground collagen particles into the high-pressure reactor.

Extraction: Treatment of loaded collagen with supercritical CO₂ at specific pressure and temperature conditions for a predetermined duration.

Depressurization: Gradual depressurization of the system to release extracted collagen from the solvent.

Collection: Collection of extracted collagen from the reactor.

Evaporation: Evaporation of residual CO₂ from the collected collagen using a vacuum.

Appendix C: Data Analysis

SDS-PAGE Analysis: Gel electrophoresis results showing the molecular weight distribution of extracted collagen samples.

FTIR Spectroscopy: Fourier-transform infrared spectroscopy data illustrating the structural characteristics of extracted collagen samples.

Cell Culture Assays: Cell viability and proliferation information gathered from cell culture studies using extracted collagen as a scaffold.