Study of biologically active peptides from plant materials: A succinct overview

Aidai Turatbekova[1*], *Dilbar* Mirzarakhmetova[1,2,3], *Nan* Shang[4], Nusratillo Toshpulatov[5], *Raykhon* Tajibayeva[6], *Nadira* Sabirova[6], *Buvzaynab* Mamarajapova[7], *Rano* Tukhtaeva[8]

[1]Department of Chemistry and Biology, Kimyo International University in Tashkent, 100121 Tashkent, Uzbekistan
[2]Tashkent State Technical University, Tashkent 100095, Uzbekistan
[3]National University of Uzbekistan named after Mirzo Ulugbek, Tashkent 100174, Uzbekistan
[4]College of Engineering, China Agricultural University, 100107 Beijing, P.R. China
[5]National Research University TIIAME, 100000 Tashkent, Uzbekistan
[6]Urgench State University, Urgench 220100, Uzbekistan
[7]Department of Ecology and Environmental Protection, Jizzakh Polytechnic Institute, 130100 Jizzakh, Uzbekistan
[8]Karshi State University, 180119 Karshi, Uzbekistan

Abstract. The study of the biological activity of various peptide compounds is a novel research topic, as peptides have extensive applications in a wide range of sectors. Biologically active peptides can be obtained from various sources, including animals, plants, and microorganisms. Plants, on the other hand, have immense promise owing to their diverse chemical structures, each of which has unique characteristics and pharmacological actions. These characteristics set the plant world apart for the development of several drugs and insecticides. Plant-derived peptides perform a wide range of biological functions, including antibacterial, anticancer, antihypertensive, and antioxidant activities. This study shows that Mass spectrometry (MS) is the primary method used to identify proteins and native peptides isolated from tissues and biological fluids. Proteomics and peptidomics are two distinct peptide analysis approaches employed in this context.

1. Introduction

Plant peptides are widely used in the food, medicine, feed production, and cosmetology industries. Currently, among the various food additives, special attention is being paid to protein preparation. This is not surprising because proteins are an important part of every structural and functional unit of the cell, occupying the first place among all macromolecules contained in a living cell. Thus, in the human and animal bodies, proteins account for 14–20% of the total mass fraction and approximately 40% of the dry matter.

Bioactive plant peptides perform a wide range of functions within the plant life cycle, in addition to non-peptide hormones, which play a role in directing plant development and advancement, creating signals to environmental stress variables, and protecting the plant from disease by pathogenic microorganisms (Figure1) [1]. During growth and development, plants interact with a vast array of pathogenic and nonpathogenic organisms. When pathogenic microorganisms interact with specific membrane receptor-like kinases in plants, an immune response is triggered, which provides protection against phytopathogens [2, 3]. It has been discovered that plants are capable of regulating the intensity of the immune response through the production of endogenous peptides known as phytocytokines. In recent years, more than a dozen phytocytokines with specific receptors have been identified. The diversity of receptors that identify pathogenic elicitors and regulatory immune peptides is a determinant of plant resistance to phytopathogens [4, 5]. Peptides that regulate growth and development have also been implicated in stress responses during phytopathogen infection. Peptides, such as antimicrobial peptides, can act as protective agents by destroying the cells of pathogenic organisms [6, 7, 8].

Proteins that are pharmaceutically inert in their natural state undergo hydrolysis to produce the majority of the bioactive peptides generated from plants that have been found. Bio-active peptides can be produced through microbial fermentation or in vitro procedures, such as hydrolysis with different proteinases, treatment at high temperatures, or treatment under harsh pH conditions [9, 10]. When extracted from precursor proteins, plant-derived bioactive peptides

*Corresponding author: turatbekova888@gmail.com

display varying biological activities depending on their peptide sequence, amino acid composition, chain length, and chemical structure. Alternative microbicides are required to combat infectious diseases, because the sharp rise in drug-resistant infections poses a challenge to traditional antibiotic therapy. Bioactive peptides exhibit antibacterial, antiviral, antifungal, and anti-parasitic properties. Comparative examination of these compounds showed that no special structural characteristics are needed to distinguish between these activities and to make classification easier [11, 12]. Cysteine and glycine residues constitute the majority of bioactive peptides, and the formation of disulfide bridges between cysteinyl residues contributes to their stability. Most have hydrophobic and cationic domains composed of charged amino acids. Because drug-resistant infections are challenging traditional antibiotic therapies due to their rapid development, alternative microbicides are needed to combat infectious diseases.

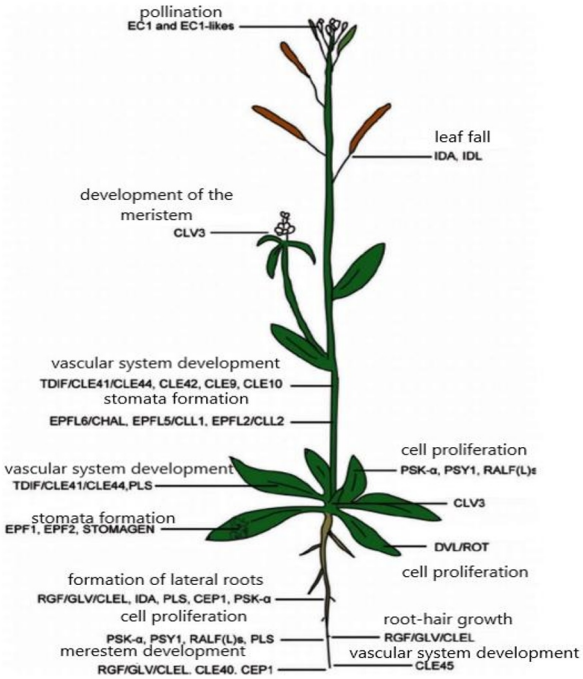

Fig. 1. Various plant peptide hormones [1]

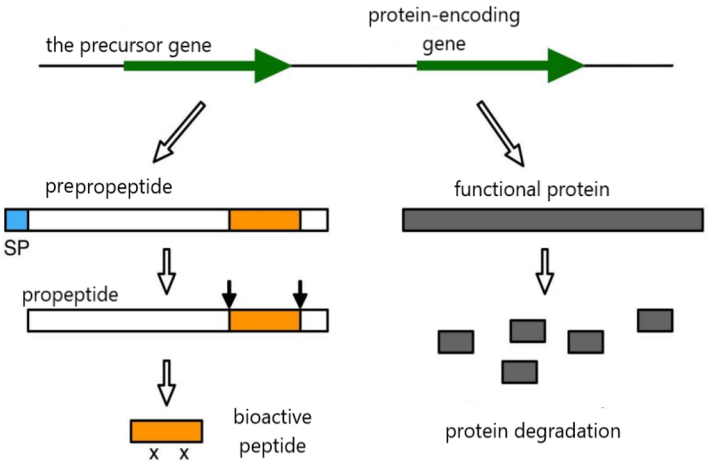

Fig. 2. Plant peptide formation during precursor protein cleavage

As bioactive peptides exhibit antibacterial, antiviral, antifungal, and/or antiparasitic properties, they can play this role. Comparative examination of these molecules shows that no special structural characteristics are needed to distinguish between these activities and to make classification easier. Cysteine or glycine residues constitute the majority of bioactive peptides, and the formation of disulfide bridges between cysteinyl residues contributes to their stability. Most have hydrophobic domains in addition to charged amino acids, which are mostly cationic [13, 14, 15].

Peptides with biological activity can be synthesized in plant cells in several ways: from non-functional precursor proteins, functional proteins, and direct synthesis on ribosomes. The majority of plant signal peptides are formed through targeted proteolysis of inactive precursor proteins, followed by post-translational modifications (Figure 2). A precursor protein with an export signal at its N-terminus is thought to pass through the endoplasmic reticulum (ER) on their way to the Golgi apparatus [16, 17] and then undergo processing to include all the necessary post-translational modifications to complete the peptide. The movement of the secreted peptides into the extracellular space is believed to be mediated by passive diffusion. Various biochemical techniques have demonstrated that the proteasomal pathway protein degradation plays a key role in the formation of a pool of such peptides.

2. Research Methods

2.1. Signaling peptides involved in plant immunity

Plants possess an innate immune system that enables them to actively resist numerous plant diseases, even in the absence of adaptive immunity. The modulation of plant immune responses is significantly influenced by peptides. Recently, it has been found that signaling and regulatory peptides, in addition to antimicrobial peptides, are also involved in adjusting the strength of protective signals. These peptides are detected by specific receptors on the surface of cells and are concealed in the structure of functional proteins or specialized precursors.

Plant signal peptides are categorized into four groups based on how they function: *phytocytokines,* which are released during stressful conditions and aid in amplifying and dispersing a danger signal among undamaged plant cells; *peptides from pathogenic organisms* that initiate immune signaling cascades in plants; *growth regulator peptides* [4, 18, 19], which are formed in the plant itself and responsible for regulating physiological processes occurring in cells both during stress and under normal conditions; and *antimicrobial peptide*s, which directly affect pathogenic organisms during invasion (Figure 3).

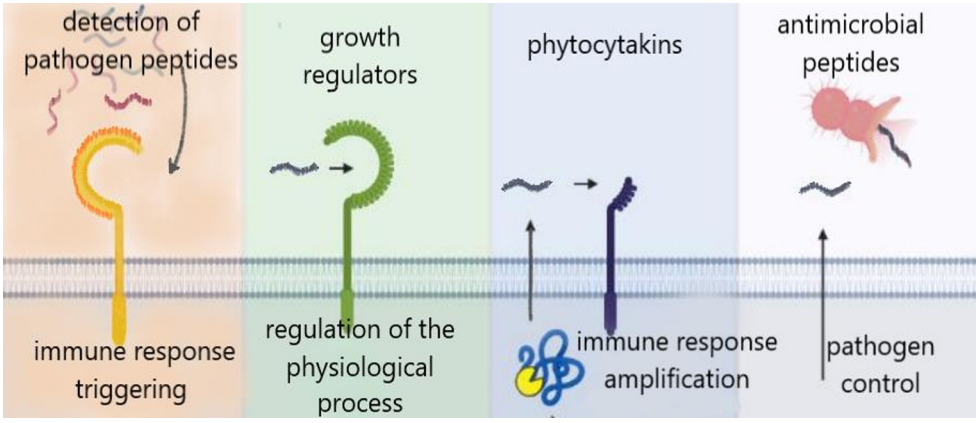

Fig. 3. Types of peptides in plant immune signaling (authors' drawing)

Growth regulator peptides include, for example, phytosulfakine (PSK), which also has antipathogenic activity and induces cell dedifferentiation and proliferation; SCR/SP11 (S-locus cysteine rich protein/S-locus protein 11), which controls pollen self-incompatibility; Clavata (CLV), which controls the growth of stem apical meristems; and CLAVATA3/EMBRYO-SURROUNDING REGION-RELATED (CLE), which promotes stomatal closure in drought-stricken environments [20, 21].

Peptides related to *phytocytokines* are frequently produced into the extracellular space and bind to a particular receptor, activating the relevant immunological signaling cascade. The expression of precursor genes for these peptides is frequently increased in response to pathogen identification by plant receptors, resulting in a stronger immunological response. One example is the PAMP-induced secretory peptide PIP. Members of the peptide families that make up the phytocytokine group are often quite numerous, and different members of the same family may exhibit quite different, even opposing, functions in plants. The RALF peptide family serves as one example of this. The RALF group of regulatory peptides impacts the growth of roots, hypocotyls, the creation of root hairs, and the elongation of pollen tubes in angiosperms, as well as the reaction to stress conditions [22, 23].

Antimicrobial peptides (AMP) are one of the most well-known types of peptides. These peptides function as vital immunological barriers that ward off pathogen attacks. Antimicrobial peptides are amphiphilic, meaning that their positively charged molecular structure interacts with the negatively charged membrane to cause selective permeability disruption or membrane destruction. Many antimicrobial peptides are active against a wide range of microorganisms, as well as phytopathogenic microorganisms, and typical examples of this group includes peptides that are antibacterial, antifungal, active against human and insect parasites. Antimicrobial peptides have a number of general

physicochemical properties, such as small molecule size, positive charge and amphiphilic structure. Plant AMPs can be divided into several families based on the similarity of amino acid sequences, the presence of cysteine residues and disulfide bonds, as well as certain tertiary structures [24, 25, 26]. The main families of cysteine-containing antimicrobial peptides from plants include defensins, thionins, hevein-inotin-like peptides, lipid transport proteins and some others. In addition, a number of other rich ones are known amino acids of antimicrobial peptides, including glycine-rich peptides (Pg-AMP1), glycine- and histidine- rich kephelins, as well as peptides less than 10 AA in length, such as Cn-AMP1 and Cr-ACP1 [13]. Peptides' antibacterial potential is predicted using in silico methodologies based on various machine learning algorithms [27, 28]. Potential antibacterial motifs within lengthy polypeptide chains of proteins can be predicted using the amino acid sequence of a protein as well as physical parameters such as charge and hydrophobicity.

2.2. Biologically active peptide analyzing methods

Mass spectrometry (MS) is the main method used to identify proteins and native peptides isolated from tissues and biological fluids. Proteomics and peptidomics are the two distinct peptide analysis approaches that are employed in this instance. Nonetheless, there exist essential distinctions among analytical techniques utilized in the identification of peptide proteins. Proteomics protein identification techniques rely on the enzymatic cleavage of proteins with certain enzymes. Such a step is necessary for the study of the negative peptides, and mass spectrometry may identify the sample instantly upon its isolation.

2.2.1. Proteomic approach

In proteomics, peptide identification is carried out using two independent processes. One is the separation of proteins based on differences in charge, more precisely, differences in visoelectric point, and the other is based on differences in size (mass) at the initial stage of research at the level of individual proteins is to obtain them in a state suitable for further analysis. The starting material can be liquid or solid. Solid substances require extraction. The extraction method depends on the type of sample and the purpose of the experiment. However, in all cases it should be considered that during the extraction process, various enzymes can act on the proteins of the sample. Since these are mainly different proteases that hydrolyze (destroy) proteins, processing, especially its initial stage should be carried out at low temperatures, for the shortest possible time and with the addition of protease inhibitors. The first stage usually involves *homogenization* of the sample (Figure 4). Most proteins, including enzymes, are localized within cells. Therefore, before isolating proteins from biological objects (animalorgans and tissues, microorganisms or plant cells), *theresearchobjectis* thoroughly crushed to a homogeneous state. Several physical procedures are utilized to destroy the cells, including homogenization with mechanical homogenizers of various designs, sonolysis, freeze-thaw, etc. [29]. The simplest technique is to use a mortar and pestle to rub the cells with aluminum oxide or abrasive powder.

Extraction occurs shortly after the biomass is homogenized from the homogenate in order to preserve the protein-soluble state. Extractants include 8-10% salt solutions (NaCl, KCl), glycerin, mild sucrose solutions (particularly for membrane protein solubilization), buffers, and organic solvents. Buffer solutions with a pH close to neutral are utilized since the medium's pH influences peptide solubility during the extraction procedure. Following extraction, the resultant extract is cleared by centrifugation to remove cellular debris [30, 31]. The particle size is an important component in controlling the rate and timing of centrifugation.

Fractionation methods include salt deposition, organic solvent deposition, and isoelectric deposition, in which protein molecules are non-denatured. There are also irreversible techniques for depositing proteins, such as profound denaturation, which results in discontinuous covalent bonds across the protein molecule and prevents the denatured protein from returning to its original state [32]. Additionally, a denaturation can be occurred as a result of pH and temperature variations. An essential step before isolating individual proteins from concentrated fraction is the separation of soluble proteins from low molecular weight components (ammonium sulfate, organic solvents). For this, dialysis and gel filtration techniques are employed.

Dialysis is the process of separating compounds with different molecular weights using a semi-permeable membrane (Figure 5). Semi-permeable membranes (cellophane) with various pore diameters are used for dialysis. This technique exploits the fact that low molecular weight compounds can easily cross a semi-permeable membrane, while proteins cannot. Since the substance to be removed is in a pure solvent, the diffusion of the latter through the membrane is guaranteed by the variations in solution concentrations on opposite sides of the membrane [33]. Protein solutions are submerged in pure solvents (water, physiological solutions, buffer solutions) and deposited in plastic bags. When the concentration of low molecular weight materials is the same on both sides of the membrane, they permeate into the solvent from the bag. It is recommended that the solvent be changed on a frequent basis to promote diffusion. Lower pressure chromatography, or desalting, has supplanted dialysis in recent times. It is necessary to employ hydrophobic sorbents for this approach.

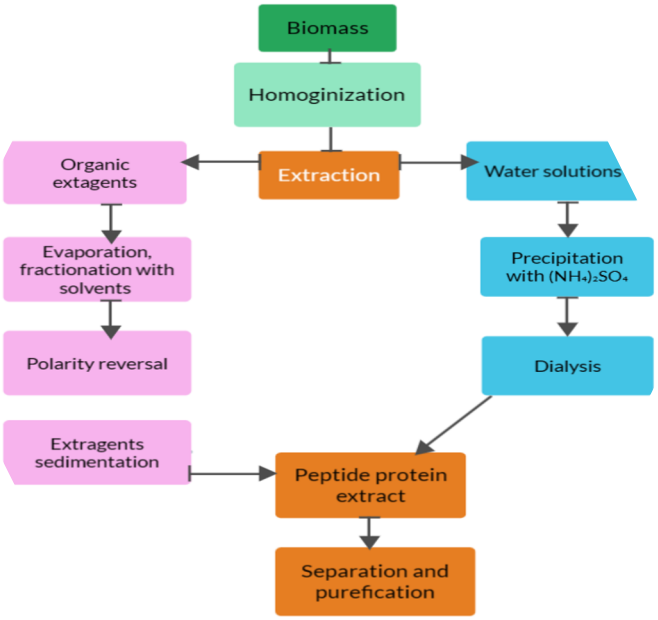

Fig. 4. General isolation type in proteomic methods (authors' work)

Protein isolation from a mixture of proteins with similar physicochemical properties is based on differences in proteins: molecular weight (ultracentrifugation, ultrafiltration, and gel filtration), charge (ion exchange chromatography), degree of adsorption of proteins and their solubility in the corresponding solvent (adsorption chromatography), and the ability of proteins to specifically interact with an affinity ligand (affinity chromatography) [34, 35].
Ultracentrifugation is high-speed centrifugation. Centrifugation separates substances based on the varied behavior of the particles in an increasing centrifugal field. Particles with varied densities, sizes, and molecular weights sink at various rates. The particle sedimentation rate was proportional to the centrifugal acceleration (g). In this situation, the protein sedimentation rates were proportional to the molecular weight (Figure 6) [36].

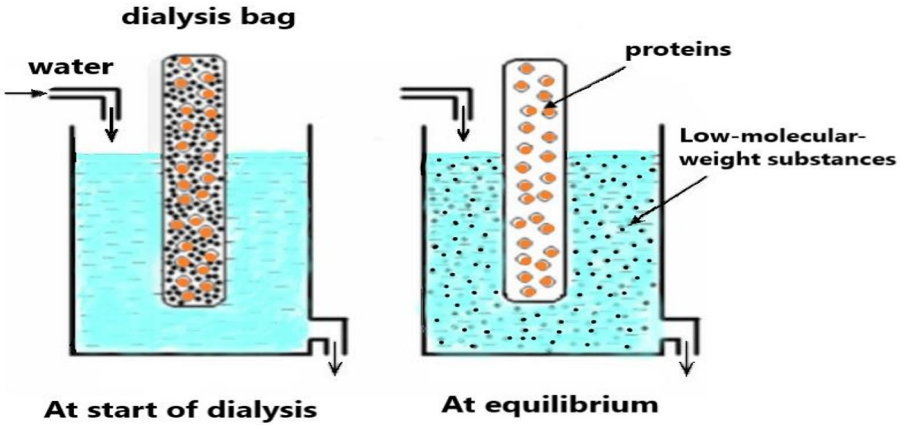

Fig. 5. Dialysis process (authors' drawing)

Gel filtration (also known as gel chromatography) is a method of separating compounds based on molecule size differences. It is a form of liquid-liquid chromatography in which separate liquids serve as both mobile and stationary phases (Figure 7) [37]. In this case, the mobile phase is the liquid that flows through the gel granule layers, whereas the stationary phase is the liquid that enters the gel granule pores. Gel filtering is done in columns packed with swelling gel particles. The stationary phase is represented by a liquid in the porous granules, which is identical to the liquid in the mobile phase flowing between the granules. During elution, molecules larger than the pores of the granules (macromolecular compounds) do not penetrate the gel granules and instead move at a high speed in the space between the granules and the solvent, leaving the gel first. Molecules smaller than the size of the granules (low-molecular-weight compounds) diffuse into and back out of the granules, slowing their elution from the column [38]. Because the degree of diffusion into the gel granules is proportional to the size of the molecules, chemicals are eluted

from the column in a decreasing order of molecular weight. The smaller the molecular weight of the molecule, the more eluent required to flush it out of the column.

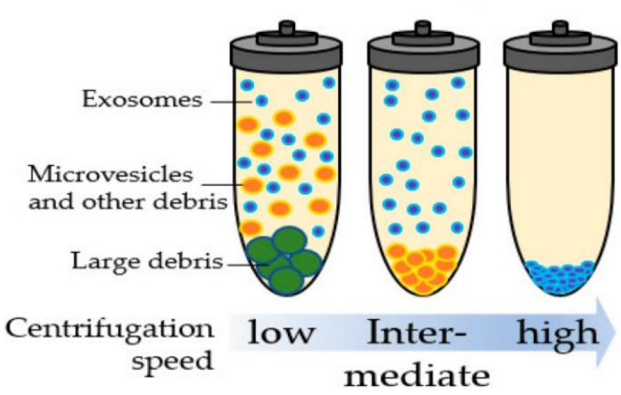

Fig. 6. Ultracentrifugation separation [36]

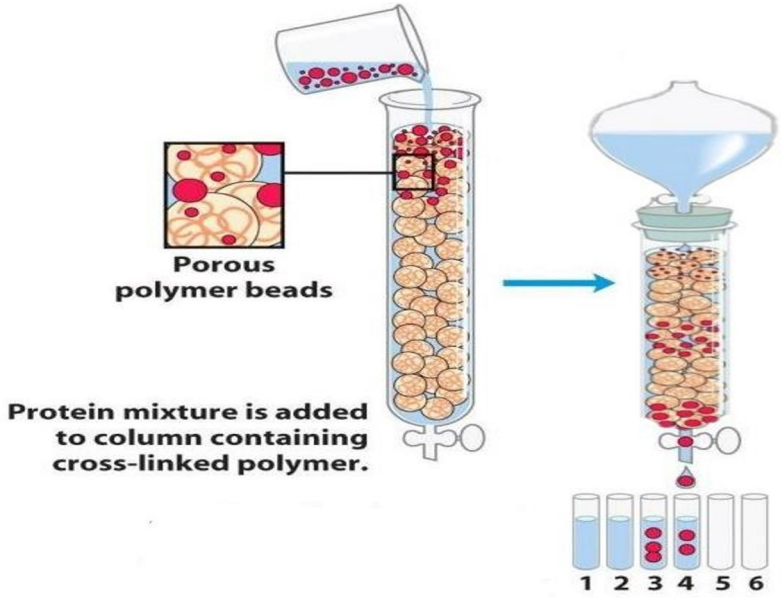

Fig. 7. Gel filtration [37]

Determination of the homogeneity of selected proteins

The homogeneity of the selected proteins is confirmed by electrophoresis. Electrophoresis involves the mobility of protein molecules in an electric field (Figure 8). A protein molecule in solution at a pH other than its isoelectric point has a restricted total charge. This is due to the variation in the functional groups of the protein chains containing amino acid residues that are partially electrolytically dissolved [39, 40]. Furthermore, protein molecules with different charges but different molecular weights differed in terms of the mass-to-charge ratio. Charged protein molecules are shifted to the opposing charged pole (cathode or anode) in response to an external electric field based on the unknown total charge. The speed at which cations move to the cathode and anions to the anode depends on the ratio of the driving force of the electric field acting on charged ions to the force of interaction between molecules and the environment (mainly frictional and electrostatic forces), which slows down the movement of ions. The electrophoretic mobility of a protein is measured as the speed of movement (cm/s) at an electric field strength of 1 V/cm. The magnitude of electrophoretic mobility corresponds to the total charge of the protein. The electrophoretic mobility of a charged molecule depends on its charge, molecular weight (size) and shape [40]. It increased as the total charge of the molecule increased and depended on the pH of the medium. The larger the molecule, the lower the mobility. This is due to the fact that larger molecules experience increased frictional forces and electrostatic interactions with the environment compared to smaller molecules.

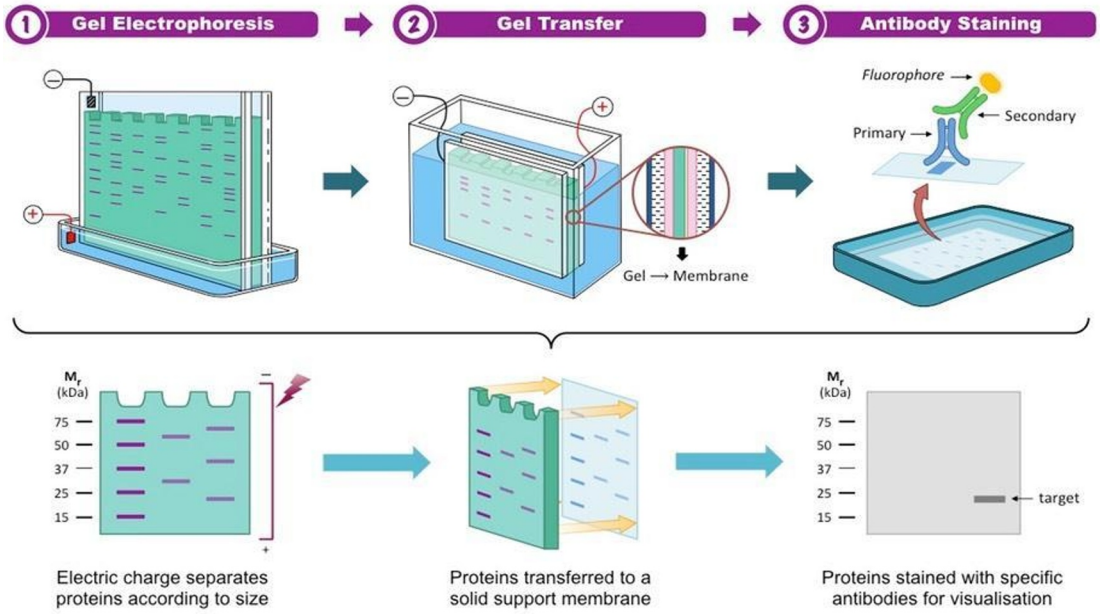

Fig. 8. Electrophoresis (source: WordPress.com)

2.2.2. The peptidomic approach

Peptidomics is a valuable approach for discovering and measuring natural peptides found in complex matrices of various origins. Natural processes, including fermentation, drying, and ripening, produce peptides using microbial and endogenous enzymes. Peptidomics uses methods such as tandem mass spectrometry (MS/MS), which has become increasingly popular recently, and high-performance liquid chromatography (HPLC) as a tool for separation to identify sample molecules. Molecular detection techniques in mass spectrometry have made it possible to forgo the conventional approach of classifying peptides according to their biological function owing to their specificity [41]. Furthermore, mass spectrometry can be used to identify specific peptides in a mixture, even if only a limited quantity of preliminary biological data about the sample is known about their structure. The main difference between peptidomics is the natural state of the molecules (endogenous pool of peptides), which is exactly the same as they exist in the biological sample from which they were isolated. One benefit of researching endogenous molecules is their ability to examine all post-translational changes, including those resulting from particular amino acid alterations or precise sites of protease cleavage. Because peptides are tiny molecules present in biological samples at extremely low concentrations, sensitive detection and identification techniques are required. Furthermore, in peptidomics research, sample separation and preparation prior to mass spectrometry is a crucial phase, the success and accuracy of which are heavily reliant on the analysis's overall performance [42]. Novel peptide analysis usually entails sample collection, peptide isolation, fractionation, MS analysis, peptide identification, and data collection. Cellular and extracellular peptide pools, on the other hand, are collections of peptides generated in tissues and biological fluids that can be collected either naturally or as a result of sample preparation artifacts. Furthermore, depending on the sample preparation procedure and MS analysis parameters, the most representative peptides with specific physicochemical attributes can be identified. Therefore, preventing sample degradation as a result of the creation of multiple peptide artifacts is one of the most crucial aspects of peptide analysis. This is particularly significant when examining tissues with the potential for rapid and active proteolysis following cell death. Several techniques have been explored to decrease nonspecific proteolysis, including quick microwave heating and the inclusion of protease inhibitors and organic solvents [43, 44]. The concern with using protease inhibitors is that they can alter the original tissue peptidome and composition of biological fluids.

Many techniques, such as protein precipitation with organic solvents, differential solubilization, ultrafiltration, and solid-phase extraction, are used to purify native peptides from various contaminants because of their high expression levels and wide dynamic range of endogenous peptides [45, 46]. The simplest and cheapest method for extracting proteins from materials is organic solvent deposition (OSD) [47]. Although peptidomics and proteomics are closely related, fundamental differences exist in the analytical methods used to identify proteins and peptides (Figure 9) [48]. Proteomic approaches rely on the inadvertent cleavage of proteins by specialized enzymes to obtain the well-known "end" of the peptide, which corresponds to the borders of ionizers in mass spectrometers. However, such a measurement is not required for peptidome recognition, and following isolation, the sample can be immediately recognized using mass spectrometry. Peptides are already present in nanomolar concentrations in cells and in the

extracellular space; thus, they are digested by large protein precursors. Early mass spectrometers had substantially lower resolution than modern spectrometers; owing to their high accuracy, the mass can only be recognized by a low-molecular-weight peptide. These characteristics make mass spectrometry a key method for flexibility in peptidomics research.

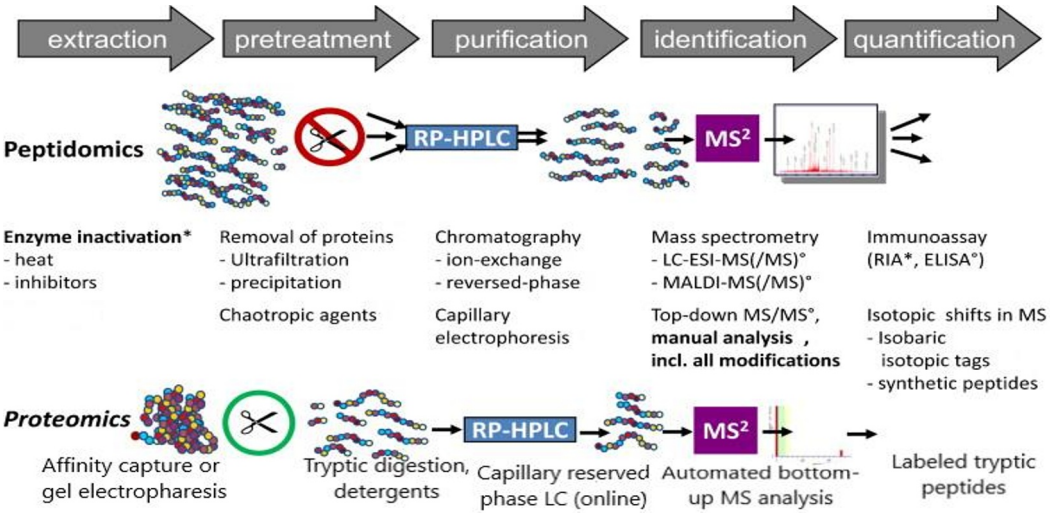

Fig. 9. Peptidomics and proteomics method's difference [48]

3. Conclusion

Depending on the features of the selected plant, multiple methods can be used to isolate specific peptides or peptide family members. Proteomic analysis is more common than peptidomics analysis. Mass spectrometry (MS) has been used almost exclusively to identify proteins and natural peptides isolated from tissues and biological fluids. However, there are significant discrepancies in the analytical methodologies used for identifying proteins and peptides. Protein identification methods rely on the enzymatic cleavage of proteins using specialized enzymes such as trypsin. Such processes are not necessary for native peptidome analysis. Once isolated, samples can be identified directly using mass spectrometry, and the use of various methods for the analysis of biologically active peptides as well as their combinations can provide additional information necessary to determine the sequence and structure of peptides.

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