CHAPTER 2

THEORETICAL

2.1 Separation Techniques

2.1.1 Affinity chromatography [7]

Affinity chromatography is based on biospecific binding interactions between a ligand chemically bound to the chromatographic packing and a target molecule in the sample. The technique is used almost exclusively to purity proteins and antibodies, although there are a few applications with peptides and nucleic acid. Immobilitized ligands may include either proteins or small molecules that interact specifically with the target of interest. A common on application is the use of antibodies as immobilized ligands.

2.1.1.1 Binding Mechanism [7]

Virtually all biological molecules interact in some selective way with some other molecules through binding at a specific site. Common examples include the binding of an antigen to an antibody, of a substrate, inhibitor or cofactor to an enzyme, or a regulatory protein to a cell surface receptor. The forced involved in the binding include the same ionic and hydrophobic interactions that cause more non-specific ion exchange or HIC binding. However, in the case of biospecific or affinity binding, the charged and hydrophobic groups are arranged on the two binding molecule or ligands in a unique orientation. Weaker forces such as hydrogen bonding also play an important role. The two ligands thus fit together very much like a lock and key, with a high drgree of specificity.

In affinity chromatography, one member of the ligand pair is immobilized (i.e. covalent coupled) as a bonded phase. Sometime a spacer arm or linker is used to place some distance between the bound ligand and the support matrix to improve accessibility. The immobilized ligand/support matrix combination forms a highly selective stationary phase that, in theory, will only bind to the other molecule (usually

a protein) of the ligand pair. In affinity chromatography, the molecules are thus separate based on their biospecific binding to the immobilized ligand.

In order to for completely specific binding to occur, it is important that the support matrix and spacer arm themselves have minimal binding interaction (nonspecific adsorption) with any of the molecules in solution. The surface of the support matrix should be very hydrophilic and have no ionic charge. Ligand leakage, which can contaminate the final product, is also very much a function of the support matrix and linking chemistry.

The binding and elution mechanism of affinity chromatography were shown in these picture

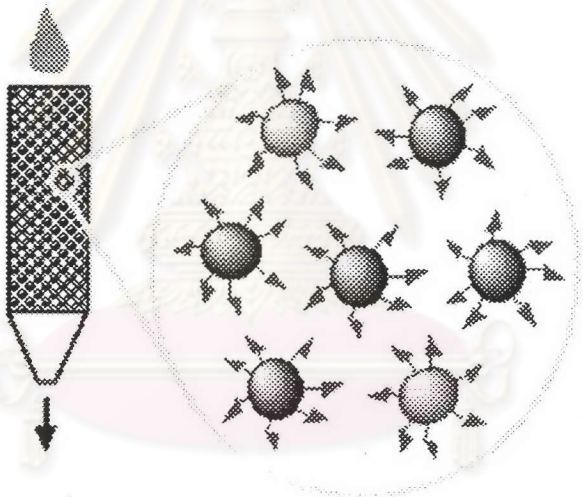


Figure 2.1 Loading affinity column.

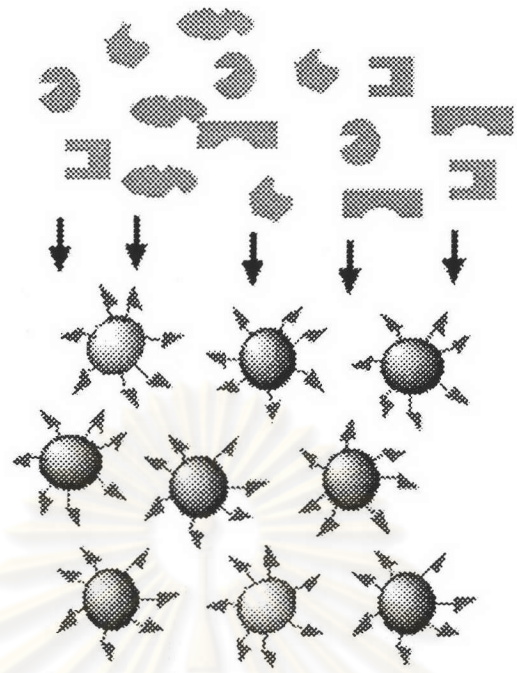


Figure 2.2 Proteins sieve through matrix of affinity beads

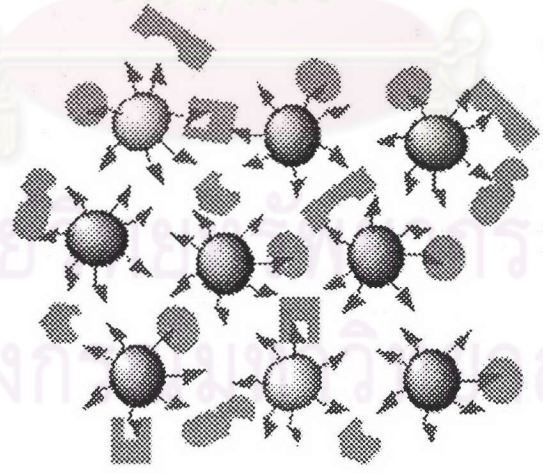


Figure 2.3 Proteins interact with affinity ligand with some binding loosely and others tightly.

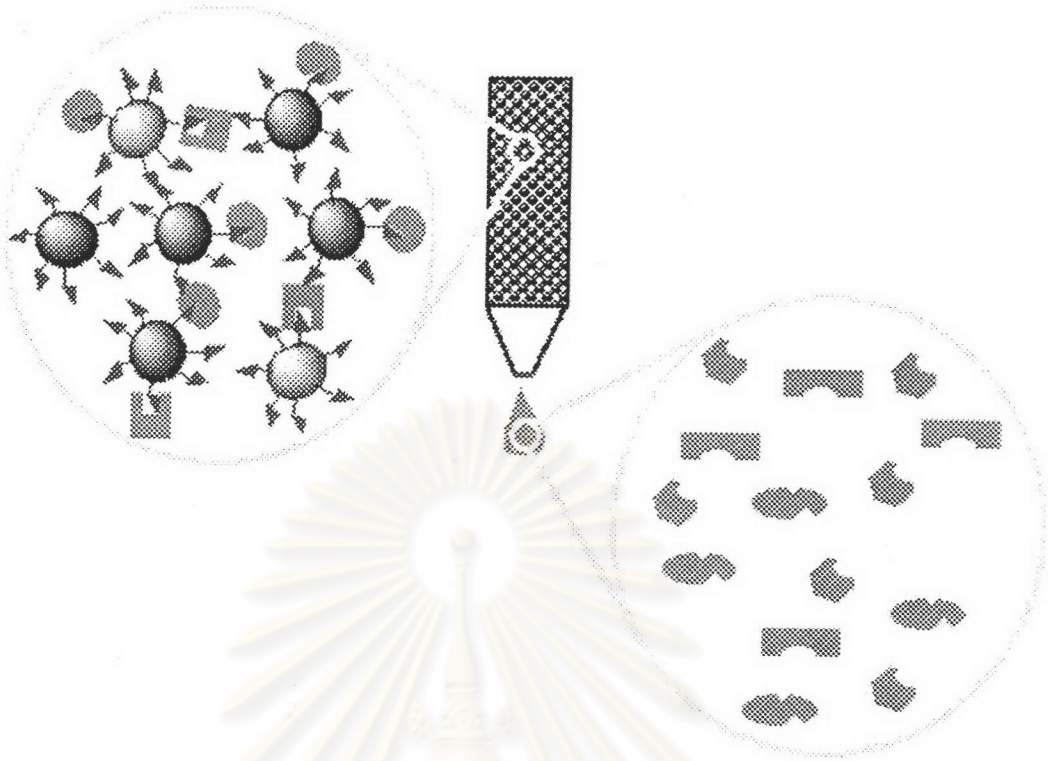


Figure 2.4 Wash off proteins that do not bind.

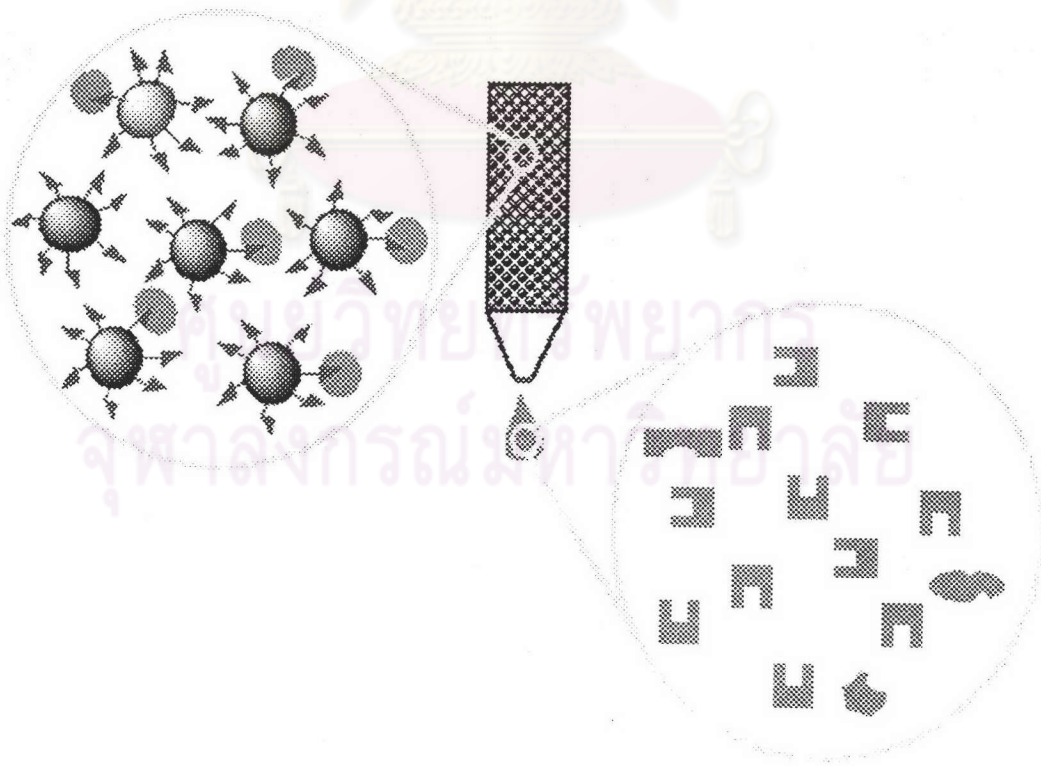


Figure 2.5 Wash off proteins that bind loosely.

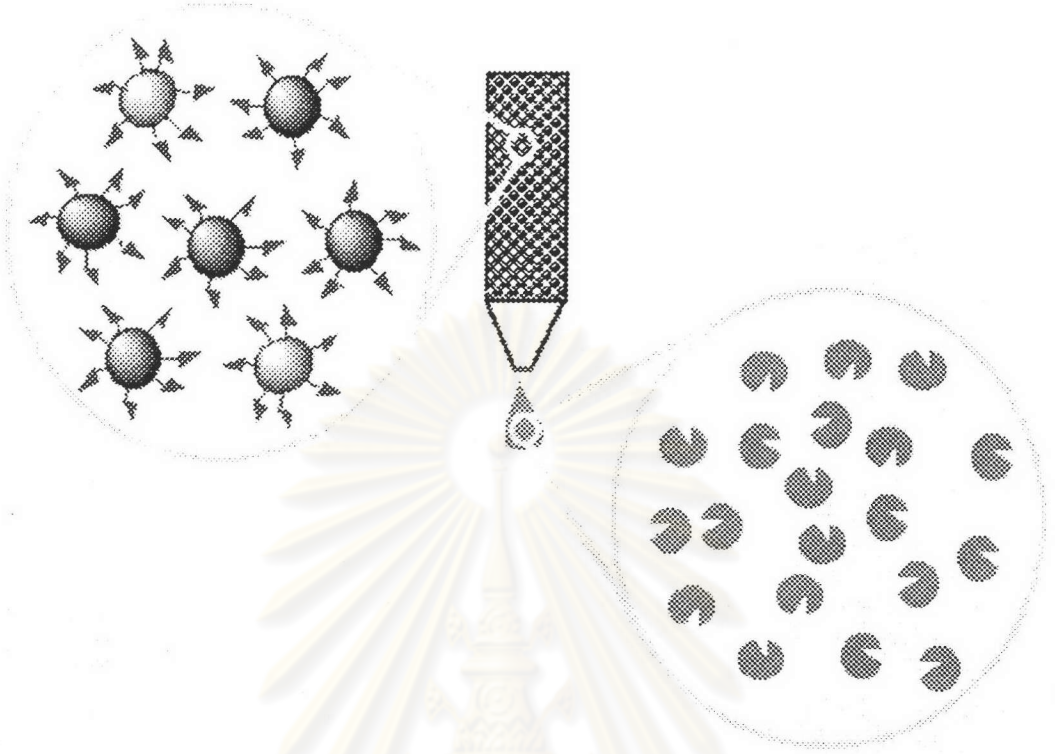


Figure 2.6 Elute proteins that bind tightly to ligand and collect purified protein of interest.

2.1.1.2 Methods of affinity chromatography [11]

Affinity chromatography techniques are not generally appropriate for the fractionation of mixture because of the very positive and specific affinity between the ligand and solute molecules. Separation may be achieved in a column where the solute molecules bind to the matrix, and, after washing to remove unwanted substances may be used in which the affinity medium is mixed with the sample solution and allowed to react. The solid-phase complex is then removed from the bulk solution by filtration or centrifugation and after washing, the purified solute is separated from the complex.

In order to elute molecules which are strongly bound to the ligand group it is necessary either to reduce their affinity for each other or to introduce molecules that are more strongly bound or in greater concentration and will therefore be able to displace the test molecules. Generally, non-specific methods are preferred and involve

altering either the ionic strength or the pH of the buffer. These will result in conformational changes in the proteins and hence their binding characteristics. Changes in the dielectric constant of the solvent caused by the introduction of organic solvents will result in altered hydrophobic bonding and again aid the dissociation of the complex. It is possible to use specific agents which complete for the binding sites, such as alternative substrates and inhibitors for enzymes.

2.1.1.3 Application for lectins [11]

Some proteins extracted from certain seeds are capable of binding compounds containing carbohydrate groups. These proteins are known as phytohemagglutinins or lectins. Affinity chromatographic media using such lectins have been used to investigate cell membrane structures and aid in the study of cell interactions. They are also used in conjunction with quantitative column chromatographic methods and in some electrophoretic separations of carbohydrate-rich proteins.

Affi-gel blue gel [1]

Affi-Gel blue affinity gel is a beaded, crosslinked agarose gel with covalently attached Cibacron® Blue F3GA dye. It contains ≥ 1.9 mg dye per ml of gel, and has a capacity for albumin binding of greater than 11 mg/ml. Affi-Gel blue gel purifies a large range of proteins from widely divergent origins. The blue dye functions as an ionic, hydrophobic, aromatic or sterically active binding site in various applications. Proteins that interact with Affi-Gel blue gel can be bond or released with a high degree of specificity by manipulating the composition of the eluent buffers. In many cases, one can also predict what will interact with the matrix and the general conditions under which binding and elution will occur.

Affi-Gel blue gel is supplied ready to use as aqueous slurry of fully hydrated gel. It is available in two convenient particle sizes: a faster flowing 50-100 mesh (150-300 μm) and a slower flowing, higher capacity 100-200 mesh (75-150 μm). The gel is also can be used with the Econo System, FPLC®, and HPLC systems.

Concanavalin A [5]

Con A Sepharose™ is Concanavalin A coupled to Sepharose 4B by the cyanogens bromide method.

Concanavalin A (Con A) is a tetrameric metalloprotein isolated from *Canavalia ensiformis* (jack bean). Con A binds molecules containing α-D-mannopyranosyl, α-D-glucopyranosyl and sterically related residues. The binding sugar requires the presence of C-3, C-4 and C-5 hydroxyl groups for ratio with Con A. Con A coupled to Sepharose is routinely used for separation and purification of glycoproteins, polysaccharides and glycolipids. Other application areas where Con A Sepharose 4B has beed used are purification of enzyme-antibody conjugates, purification of IgM, isolation of cell surface glycoproteins from detergent-solubilized membranes,

2.1.2 Gel filtration [18, 23]

Gel filtration depends on the fact that the particles of column material are porous, with pores of more or less defined size. Large molecule cannot enter these pores and thus excluded (recall that exclusion chromatography is an alternative name for gel filtration); however, small molecule can penetrate the pores, hence the alternative name gel permeation chromatography (Hagel 1989; Stellwagen 1990a; Cutler 1996a). Consequently, large molecules are swept past the particles of column material by the flow of elution buffer, whereas smaller molecules diffuse into the particles and, being retarded, are transported through the column more slowly than larger molecules. The volume occupied by the elution buffer external to the gel matrix is termed the excluded volume; it can be determined by measuring the volume required to elute a large tracer molecule from the column which is not able to penetrate into the gel pores.

An ideal matrix for gel filtration should consist of particles of a hydrophilic polymer that is as inert as possible, as rigid as possible, uncharged and of uniform size. Suitable materials are naturally occurring polymer, such as agarose or dextran, which have been stabilized by chemical cross linking, and also synthetic polymers such as polyacrylamide. These materials are avalible as spherical particles of different

diameter and pore sizes; the pore size determines the range of optimal molecule weight separation.

One of the most popular gel filtration materials is Sephadex, supplied by Pharmacia, which consist of detran cross-linked by epichorhydrin; this is supplied in dried from which must be pre-swellen in water taken up by the Sephadex particles depends on the degree of cross-linking, as dose the time taken to complete the process; swelling is more rapid at higher temperature. The porosity of the material determines the exclusion limit: G-10, G-15, G-25 and G-50 are suitable for peptide separations, and G-75, G-100, G-150 and G-200 for proteins and other macromolecules.

2.1.2.1 Separation Mechanism [7]

Gel filtration depends on the fact that within each particle of the stationary phase there is a distribution of pore sizes. For small enough molecules, the pores are so large that the molecules can penetrate all of the internal volume of the particle. If the molecules are large enough, the pores are so small that the molecule is completely excluded from the internal volume. Molecules in between will have access via diffusion to a portion of the internal volume but will be excluded by the smaller pores from the rest. In gel filtration, molecules are thus separated based on size or molecular weight.

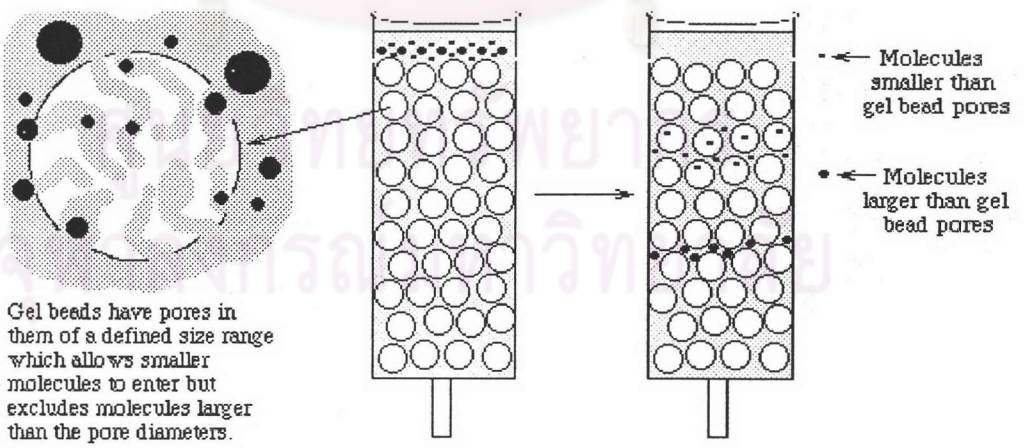


Figure 2.7 Separation mechanism of gel filtration

2.1.3 Reversed-Phase Chromatography [7]

Reversed-phase chromatography is the most common chromatographic mode for analysis of any type of molecule, as well as for preparative separations of small molecules, peptides and oligonucletides. Like hydrophobic interaction chromatography, reversed-phase chromatography separate molecules based on differences in hydrophobicity.

Binding Mechanism

The bonded phase in reversed-phase is an extremely hydrophobic or nonpolar surface. The mobile phase is polar, usually water or an aqueous solution. Nonpolar or hydropholic molecules bind preferentially to the stationary phase and the polar molecules remain in he mobile phase. The separation is thus based on the principle that oil and water do not mix.

In the case of proteins, the hydrophobicity of the stationary phase is so extreme that the protein often loses its three dimensional shape and denatures. This is because in aqueous solution, a major force holding the protein in its correct shape is the tendency for the hydrophobic amino acids to cluster at the core of the protein, where they can exclude water. When exposed to the reversed-phase surface, the protein may unfold allowing internal hydrophobic groups to bind.

This is not the case in hydrophobic interaction chromatography where the high salt concentration and weakly hydrophobic packing surface tend to keep the protein stable in its correct 3-D conformation. For this reason, the selectivity and binding characteristics of these two modes may be very different, even though they are both separating based on hydrophobicity.

Elution Method

Reducing the polarity of the mobile phase allows the hydrophobically bound molecules to partition off the bounded phase surface, causing elution from a revered-phase column. This is usually done by adding a water-miscible organic solvent such as acetonitrile or an alcohol (methanol or isopropanol are the most common)

Elution is most often performed in a continuous gradient. Reversed-phase separations (especially with peptides) often show the best resolution with a very shallow or "near isocratic". This contrasts with ion exchange or hydrophobic interaction, where the increased band spreading overcomes any benefit from increased difference in retention in very shallow gradients. In reversed-phase there is usually a very narrow range in solvent concentration over which a given molecule goes from no elution at all to no binding at all. Very slight shifts in solvent concentration can greatly affect retention, so a shallow gradient is usually more reproducible than true isocratic elution.

2.1.4 Sodium dodecyl sulfate polyacrylamide gel electrophoresis [26]

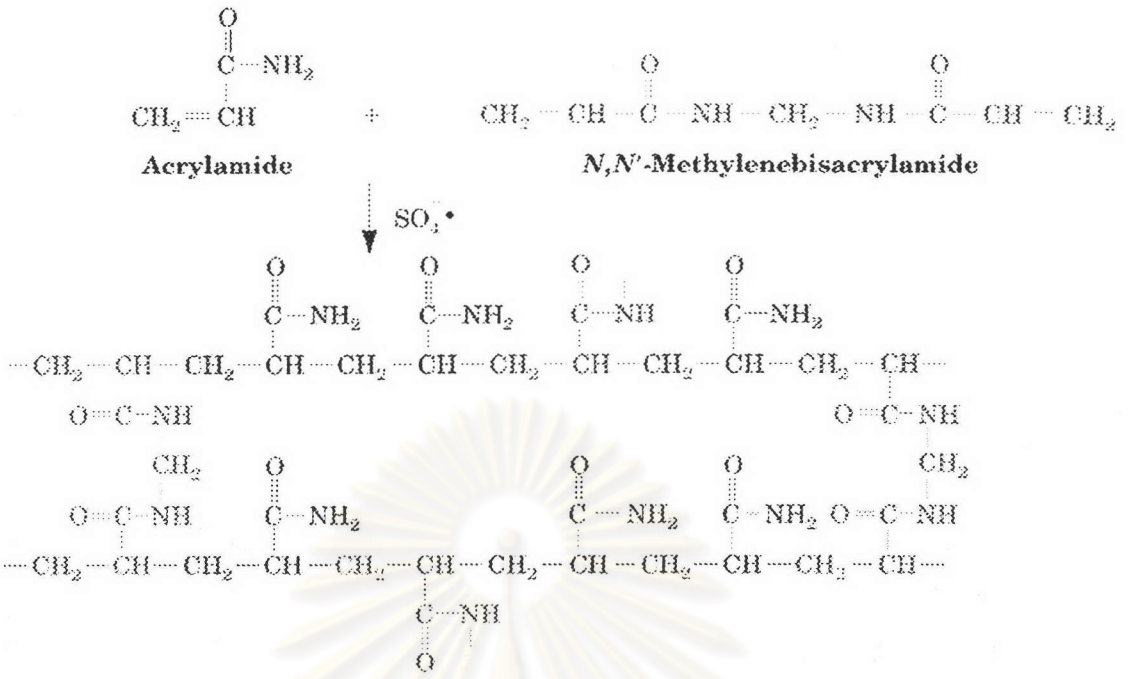
Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is the most widely used method for analyzing protein mixtures qualitatively. It is particularly useful for monitoring protein purification and, because the method is based on the separation of proteins according to size. The method can also be used to determine the relative molecular mass of proteins.

2.1.4.1 Components of SDS-PAGE

There are two major important components are as follows:

1) Polyacrylamide [26]

Cross-linked polyacrylamide gel are formed from the polymerization of acrylamide monomer in the presence of smaller amounts of *N, N'*-methylenebisacrylamide (normally referred to as bis-acrylamide). Note that bis-acrylamide is essentially two acrylamide molecules linked by a methylene group,and is used as cross-linking agent. Acrylamide monomer is polymerized in a head-to-tail fusion into long chains and occasionally a bis-acrylamide molecule is built into the growing chain, thus introducing a second site for chain extension. Preceding in this way a cross-linked matrix of fairly well-defined structure is formed.



Schem 2.1 The formation of a polyacrylamide gel from acrylamide and bis-acrylamide

The polymerization is initiated by the addition of ammonium persulpate and the base *N, N, N', N'*-tetramethylenediamine(TEMED). TEMED catalyses the decomposition of the persulfate ion to give a free radical (i.e. molecule with an unpaired electron)

Free radicals are highly reactive species due to the presence of an unpaired electron that needs to be paired with acrylamide monomer molecule, forming a single bond by sharing its unpaired electron with one from the outer shell of the monomer molecule. This therefore produces a new free radical molecule, which is equally reactive and will attack a further monomer molecule. In this way long chains of acrylamide are built up, being cross-linked by the introduction of the occasional bis-acrylamide molecule into the growing chain. Oxygen removes free radicals and therefore all gel solutions are normally degassed prior to use.

Polyacrylamide gels are transparent and flexible, yet relatively strong and resilient. They are chemically inert and are compatible with numerous buffers salt and detergent. Overall, protein mobility through polyacrylamide gels is proportional to the pore size, which is a function of both the acylamide concentration (%T) and that of the bis-acrylamide cross-linked (%C). In general, the pore size is inversely proportional to $\%T^{4}$.

$$\%T = \frac{\text{acrylamide(g) + bis-acrylamide (g)}}{\text{100 ml}} \times 100\% \qquad (1)$$

$$\%C = \frac{\text{bis-acrylamide (g)}}{\text{acrylamide(g) + bis-acrylamide (g)}} \times 100\% \qquad (2)$$

The composition of any given polyacrylamide gel is described by two parameter, %T and %C. The %T value represents the total concentration of monomer to produce the gel, and %C is the percentage of the total monomer which is the cross-linking agent. For any given total monomer concentration, the effective pore size, stiffness, brittleness, light scattering, and swelling properties of the polyacrylamide gel vary with proportion of cross-linker used.

Linear Range of Separation for Proteins
On SDS-Polyacrylamide Gels

Percent Acrylamide (19:1 bis-acrylamide)	Size Range (kilodaltons)
15	15-45
12.5	15-60
10	18-75
7.5	30-120
5	60-212

2) Sodium dodecyl sulfate [4]

Sodium doecyl sulfate (SDS) is an anionic detergent that is used to denature proteins, giving them all the same conformational properties, and to prevent protein interactions during electrophoresis. Sodium dodecyl sulfate also masks the intrinsic protein charge and gives all proteins a similar net negative charge, so that electrophoretic migration is towards the anode. In general, sodium dodecyl sulfate coats proteins with a uniform negative charge and constant charge-to –mass ratio. Hence, in an electric field SDS-coated proteins experience the same field strength and migrate at identical intrinsic rates towards the anode. Proteins of different size are subjected to different degrees of sieving by the gel, however, and the mobility of a protein is in inversely proportional to its size.

2.1.4.2 One-dimensional gel electrophoresis [26]

The one-dimensional gel elctrophoresis has two parts. The first part is stacking gel. The stacking gel is used to concentrate sample proteins in order to be a sharp band before it go to the second part or separating gel. The stacking gel has a large pore size, which the proteins can move freely under the electric field. Then negatively charged protein-SDS complexes continue to move towards the anode through the second part which is separating gel. The smaller proteins are more easily pass though the pore of gel, whereas large proteins are successively retarded by frictional resistance due to the sieving effect of gel.

2.1.4.3 Two-dimensional gel electrophoresis [13]

The first dimension is the isoelectric focusing gel. This gel,proteins contained in it, is then saturated with SDS and transferred to the molecular weight gel for the second dimension. The resulting gel has the proteins separated according to pI across the x-axis of the 2D display and according to molecular weight across the y-axis. By convention, such gels are displayed with acidic side of the gel oriented to the left, the basic side of the gel oriented to the right, and protein molecular weight decreasing from the to the bottom of the gel. The power of 2D electrophoresis is its ability to separate complex mixture. One should remember, however, that 2D electrophoresis is

an analytical method as well, measuring the molecular weight, isoelectric point, and relative amount of a protein in the mixture.

2.1.4.4 Protein detection [15]

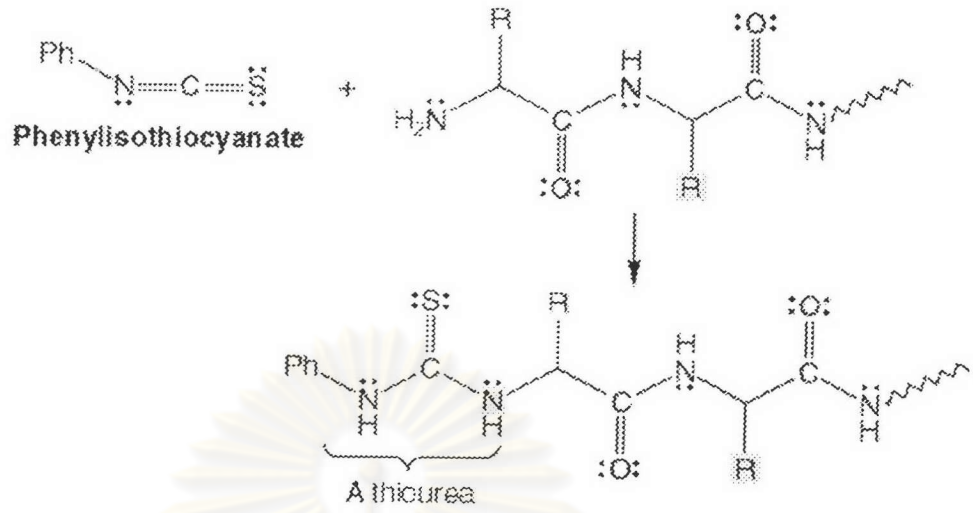
After the electrophoresis run is complete, the gel must be analyzed qualitatively or quantitatively to answer analytical or experimental questions. Because most proteins and all nucleic acids are not directly visible, the gel must be processed to determine the location and amount of the separated molecules.

The mostly applied methods in the detection of separated proteins from gel electrophoresis are Coomassie blue staining and Silver-staining. Silver staining is the most sensitive non-radioactive method (below 1 ng). Silver staining is a complex, multi step process utilizing numerous reagents for which quality is critical. Coomassie blue staining, although 50- to 100- fold less sensitive than silver staining, is simple method and more quantitative than silver staining. Coomassie blue staining is preferable when relative amounts of protein are to be determined by densitometry. For another method used to detect protein in general laboratory such as Autoradiography and Fluorography, Negative Zinc, Fluorescent staining etc.

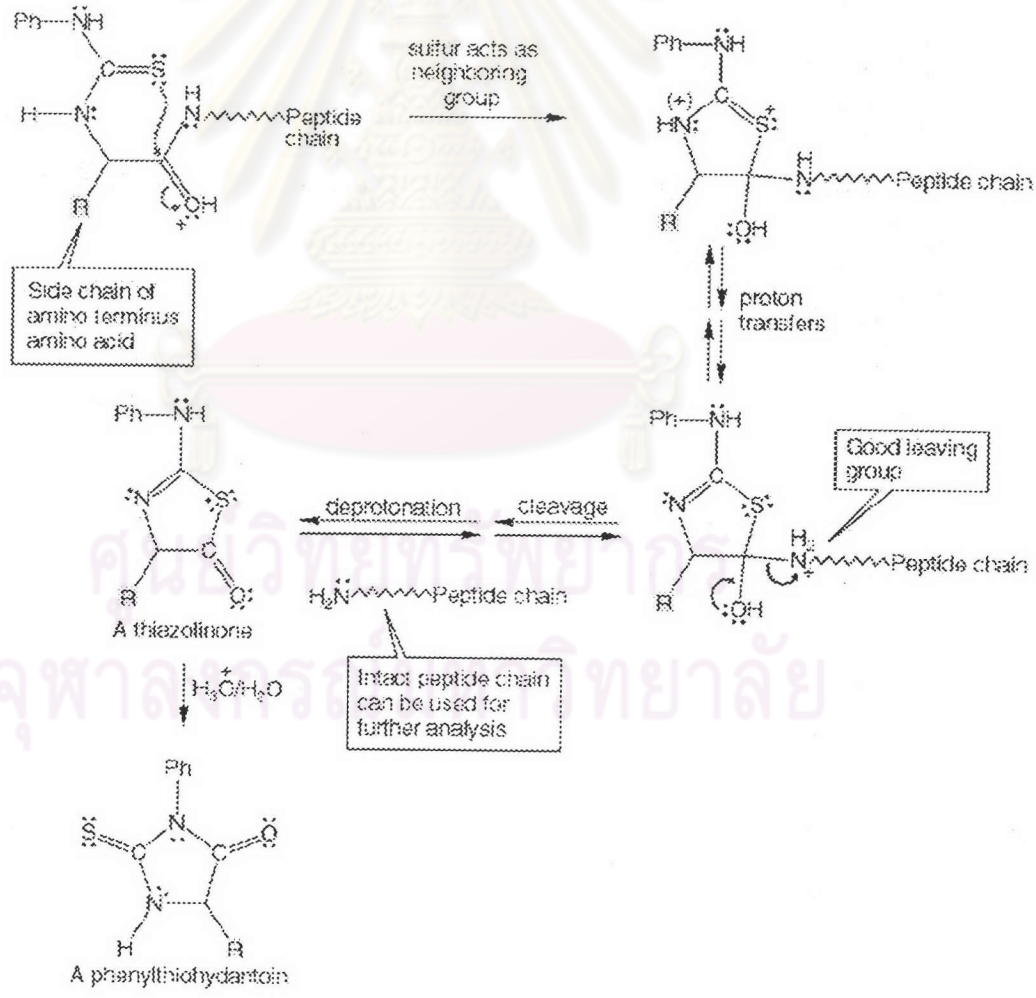
2.2 Protein Identification technique

2.2.1 The Edman Reaction [13]

The chemical reaction used in the Edman degradation of proteins was first described by Edman in a series of papers published beginning in the 1950s. In the Edman reaction, phenylisothiocyanate reacts with the N-terminus of the protein to from a cyclic intermediate that facilitates hydrolysis of the adjacent amide bond. The effect of this reaction is to specifically remove the N-terminal amino acid from the protein and thereby generate a cleaved derivative of that amino acid and a protein that is shortened by one amino acid and has a new N-terminus. The term 'degradation' suitably describes the effect of repeated applications of the Edman reaction to repetitively degrade the protein amino acid-by-amino acid from the N-terminus. At each application or cycle of the Edman reaction, the cleaved amino acid can be recovered and identified by appropriate analytical methods.



Schem 2.2 phenylisothiocyanate reacts with the N-terminus of the protein



Schem 2.3 Cyclic degradation of peptides based on the reaction of phenylisothiocyanate

2.2.1.1 Edman Degradation in Proteomic Research [13]

As a result of more than 40 years of development, current automated Edman sequenators are completely automated high-sensitivity instrument systems. These systems can routinely detect and identify as little as 0.2 pmol to 1 pmol of amino acid in a given cycle and carry out > 20 cycles with 1 to 5 pmol of protein. Higher sensitivities may be seen in non-routine applications. All reagent delivery steps; all reaction steps, including the removal of reagents; and transfer of the cleaved, derivatized amino acid to the amino acid analyzer are automated, which allows these analyzers to operate continuously with only cursory monitoring by the operator. Further, the data interpretation in an Edman degradation experiment is also a generally straightforward process that is well-suited to computerized qualitative analysis approaches.

Edman degradation should remain a part of modern protein sequencing experiment for several reasons. Most important to note, Edman degradation can be applied to intact proteins. The ability to directly sequence the N-terminus of electroblotted proteins (for example) eliminates the need for the digestion steps that are used internal sequencing experiments. The sensitivity of Edman degradation is good enough so that the amounts of protein needed for direct N-terminal analysis are produced in many routine experiments. Finally, the ease of operation of the instrument and the clarity of the data it produces add to the continued utility of the technique.

However, Edman degradation has a number of serious shortcomings, particularly relative to tandem mass spectrometry, that make it unsuited for proteomic work. The most significant Shortcoming is that the time required to complete each cycle is about 45 min, which is a length of time that allows the analysis of no more than two to three sample per day. Further, most proteins have blocked N-termini and some investigators suggest that the reagents involved in gel electrophoresis may exacerbate this problem. When the N-terminus of a protein is blocked, no data are produced and the sample is lost. Finally, whereas the sensitivity of Edman degradation is acceptable for the direct N-terminal analysis of intact proteins electroblotted and detected by Coomassie staining, it is not good enough for analysis

of the amounts of protein detectable by silver-staining of gels. Furthermore, the sensitivity of Edman degradation is also not good enough for routine internal sequencing experiments at even the Coomassie level because of the need to isolate individual peptides and the poor recovery of peptides through these procedures.

In summary, Edman degradation is an outstanding analytical method for sequencing proteins and it continues to contribute to the field of protein biochemistry. It has, however, inadequate performance in a number of critical areas, a characteristic that makes it generally unsuitable for proteomic work.

2.2.2 Mass spectrometry [13]

Although mass spectrometry can be, and is, used to characterize a wide variety of analytes, the discussion of mass spectrometry presented in this paragraph focuses on only one type of analyte-peptides produced by an enzymatic digestion of proteins. Useful peptides produce by these digests have masses approximately 500 Da to 2000 Da and are available for analysis in dilute, aqueous solution.

2.2.1.1 An overview of mass spectrometry [13]

A block diagram of a basic mass spectrometry is shown in Figure 2.8 As seen in this figure; mass spectrometers have seven major components: a sample inlet, an ion source, a mass analyzer, a detector, a vacuum system, an instrument-control system, and a data system. Detail of the sample inlet, ion source, and mass analyzer tend to define the type of instrument and the capabilities of that system. Details of the other components, although important, tend to remain in the background of instrument operation. The instruments used in the experiments described in this volume are composed of combinations of the inlets, ion sources, and mass analyzers summarized to produce four basic instrument configurations.

1. Capillary-column liquid chromatography-electrospray-tandem quadrupole mass spectrometer.

2. Capillary-column liquid chromatography-electrospray-ion trap mass spectrometer

3. Capillary-column liquid chromatography-electrospray- quadrupole-time-of-flight tandem mass spectrometer

4. Direct-pole matrix-assisted laser desorption/ionization time-of-flight mass spectrometer

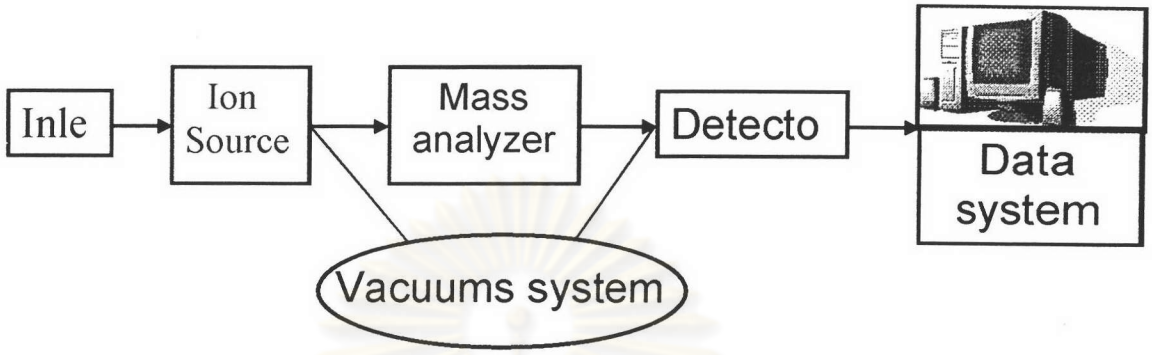


Figure 2.8 A block diagram of mass spectrometry

The basic process associated with a mass spectrometry experiment encompass the generation of gas-phase ions derived from an analyte, and the measurement of the mass-to-charge ratio (m/z) of those ions. Studying an analyte as a gas-phase ion gives two fundamental characteristics of gas-phase ions can be precisely controlled in electromagnetic fields that contain the ions for study and can be manipulated to probe the movement of the ion in the field. Because the details of this movement are in some way proportional to m/z and therefore the mass of an analyte. Details of the different mass-analysis techniques and the ionization techniques being used determine factors such as the precision and accuracy of m/z measurement, m/z resolution, and the m/z range of the analyzer. The second fundamental characteristic is that the use of gas-phase ions produces the sensitivity of mass spectrometry. The precise movement of ions in electromagnetic fields that allows m/z to be measured also provide for ion containment and focusing. During the course of m/z measurement, ions are transmitted with high efficiency to particle detectors that record the arrival of those ions. These arrivals are detected with high sensitivity due to a combination of low background signals and the efficient generation of secondary electrons that can subsequently be multiplied by factors of 10^5 and greater. Maintaining the precision of these movements and therefore the sensitivity of the analysis requires expert operation of the mass spectrometer system.

2.2.1.2 Ionization method [13]

A fundamental challenge to the application of mass spectrometry to any class of analytes is the production of gas-phase ions of those species, and difficulties in producing gas-phase ions can prevent mass spectrometric analysis of certain classes of molecules. This situation was once the case with protein and peptides. The first techniques that were applied, electron ionization and chemical ionization, are two step processes in which the analyte is vaporized with heat and ionization occurs once the analyte is in the gas phase. This vaporization step limited mass spectrometric sequencing experiments to the analysis of small peptides, usually to a maximum of 4 to 5 amino acids. Further, these peptides had to be derivatized to minimize polarity and to give them sufficient volatility. The analysis of proteins was simply not possible, and similar problems were encountered with other classes of polar molecules.

The ionization methods most commonly used to volatized and ionize the proteins or peptides for mass spectrometry is Matrix-Assisted Laser Desorption/ionization (MALDI)

2) Matrix-Assisted Laser Desorption/Ionization[10]

Matrix-Assisted Laser Desorption/Ionization (MALDI) was the first described by Karas and Hillenkamp in 1988. MALDI has significantly revolutionized the approaches to the study of biopolymer and provide a unique opportunity to apply mass spectrometry to analysis of proteins and other biomolecules. The key of this technique is mixing of sample with a matrix.

Mechanism of Desorption and Ion Formation [6]

The mechanism of MALDI is believed to consist of three basic steps:

1) Formation of a 'Solid Solution': It is essential for the matrix to be in access thus leading to the analyte molecules being completely isolated from each other. This eases the formation of the homogenous 'solid solution' required to produce a stable desorption of the analyte.

2) Matrix Excitation: The laser beam is focused onto the surface of the matrix-analyte solid solution. The chromaphore of the matrix couples with the laser frequency causing rapid vibrational excitation, bringing about localized disintegration of the solid solution. The clusters ejected from the surface consist of analyte molecules surrounded by matrix and salt ions. The matrix molecules evaporate away from the clusters to leave the free analyte in the gas-phase.

3) Analyte Ionization: The photo-excited matrix molecules are stabilized through proton transfer to the analyte. Cation attachment to the analyte is also encouraged during this process. It is in this way that the characteristic $[M+X]^+$ (X= H, Na, K etc.) analyte ions are formed. These ionisation reactions take place in the desorbed matrix-analyte cloud just above the surface. The ions are then extracted into the mass spectrometer for analysis.

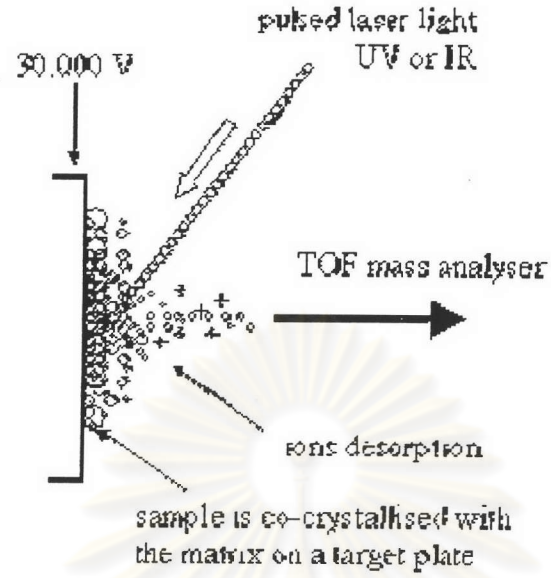
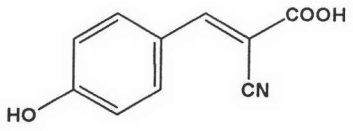
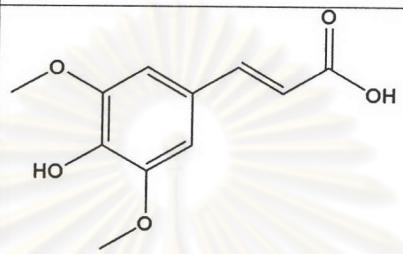
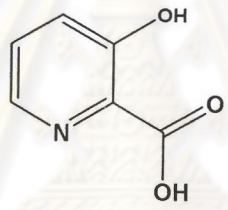
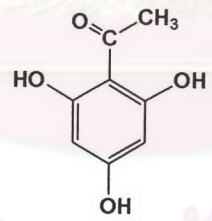
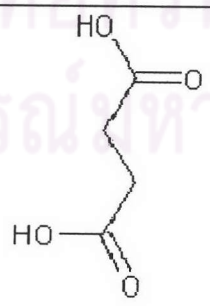


Figure 2.9 A schematic diagram of the mechanism of MALDI.

The characteristics of a typical MALDI matrix [6]

- A strong light absorption property at the wavelength of the laser flux.
- The ability to from microcrystal with the sample.
- A low sublimation temperature, which facilitates the formation of an instantaneous high-pressure plume of matrix-sample material during the laser pulse duration.
- The participation in some kind of a photochemical reaction so that the sample molecules can be ionized with high yields.

Table 2.1 The matrices used in MALDIMS [6]

Matrix	Structure	Applications
∝-cyano-4-hydroxycinamic acid		Peptides,polypeptides
Sinapinic acid		Proteins,polypeptides
3-Hydroxypicolinic acid (HPA)		oligonucleotides
2,4,6-trihydroxyacetophenone		oligonucleotides
Succinic acid		oligonucleotides

2.2.1.3 Mass analyzer [13]

Mass anslysis in mass spectrometers determines the m/z of ion derived from the analyte. The unit for m/z is the Thomson although many scientists use "m/z" as a unit –less ratio. One must take care with the interchangeable use of "mass" and "m/z", because these values will not be the same for any ion that is multiply charged. It is critical to remember this distinction when considering any mass spectrum, including a product ion spectrum, particular when electrospray ionization is being used.

Time-of-flight [6, 10]

A time-of-flight (TOF) mass spectrometry is one of the simplest mass-analyzing devices. Since the 1990s, it has reestablished itself as a mainstream technique and is becoming increasingly useful in meeting the demands of contemporary research in biomedical sciences.

Principle of operation

A TOF mass spectrometer behaves as a velocity spectrometer, in which ions are separate on the basis of their velocity differences. A short pulse of ions, after exiting the source, is dispersed in time by allowing it to drift in an FFR of a long flight tube. The principle behind the mass analysis is that after acceleration to a constant kinetic energy (equal to zV, where z is the charge on the ion and V the accelerating potential), ions travel at velocities,υ, that are an inverse function of the square root of their m/z values:

$$\upsilon = \left(\frac{2zV}{m} \right)^{1/2}$$

The lighter ions travel faster and reach the detector placed at the end of the flight tube (of length L) earlier than do the heavier ones. Thus, a short pulse of ions, is dispersed into packets of isomass ions (Figure 2.10). Therefore, mass analysis of ions that enter the flight tube can be accomplished by determining their time of arrival given by

$$t = \frac{L}{\upsilon} = L\left(\frac{m}{2zV}\right)^{1/2}$$

In order to convent the time spectrum into a mass spectrum, the instrument is mass calibrated by measuring the flight times of two different known mass ions.

A primary requirement in the operation of a TOFMS is that all ions enter the flight tube precisely at the same time. Generating ions in short fulfills this condition. In this respect, TOF instruments are well matched to ^{252}Cf-plasma desorption (PD) and MALDI ion sources. The continuous ion beam sources (e.g. electron ionization and ESI), however, can be coupled with a TOF mass spectrometer, but only after conversion of the generated ions into discrete packets. Pulsing of the accelerating potential converts a continuous ion beam into discrete ion packets.

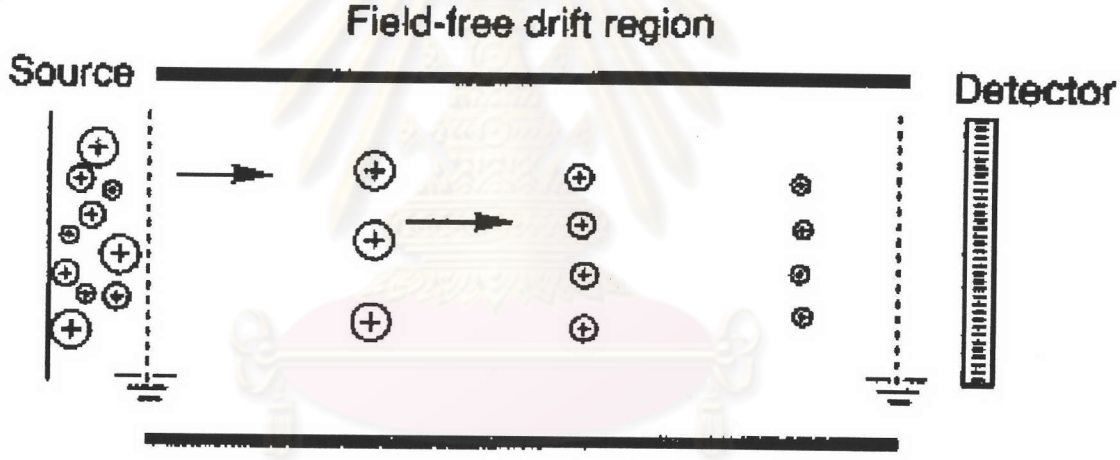


Figure 2.10 Principle of the mass separation by a time-of-flight mass analyzer.

2.3 Protein identification by database searching [9]

2.3.1 The Protein Sequence Databases

Protein identification is made by comparing the mass spectrometric data with the information that has been gathered into the sequence databases.

- SWISS-PROT (www.expasy.ch/sprot-top.html) is a database of annotated protein sequence; it also contains addition information on function of the protein, its domain structure, posttranslational modification, etc.

- TrEMBL (www.expasy.ch/srs5/) is a supplement to SWISS-PROT, which contains all protein sequences, translated from nucleotide sequences of the EMBL database.

- NCBInr (www.ncbi.nLm.nih,gov/dbEST/) is a database containing sequences translated from DNA sequences of GenBank and also sequences from PDB, SWISS-PROT and PI database.

2.3.2 Protein Identification Using Peptide Mass Mapping [10]

The peptide mass mapping usually performed using MALDI-MS. The method comprises protein digestion, MALDI/TOF analysis and sequence database search algorithms. The protein of interest can be enzymatically or chemically cleaved into its constituent peptides. The enzymatic digestion offers several advantages, including high specificity, minimal side reactions and good cleavage. The most common used enzyme is trypsin, which specifically cleaves protein on the C-terminal side of Lys and Arg, except those bonds are to proline (P) residues (K-P or R-P bonds). There are two types of digestion, in-gel digestion and in-solution digestion. The in-gel digestion methodology has become routine for proteins separated by 2D electrophoresis. Briefly, the in-gel digestion steps are protein spot picking, destaining, reduction and alkylation, tryptic digestion, and extraction tryptic peptides for analysis. For protein separated by other techniques (not gel electrophoresis), the digestion method is carried out by digest proteins in solution, so called "in-solution digestion".

For peptide, mass fingerprinting has several programs available to use. There is MASCOT at www.matrixscience.com, profound at www.prowl.com and MS-FIT at www.prospector.ucsf.edu/. The four important parameters for data search are peptides mass list, the cleavage agent, error tolerance (mass accuracy) and knowledge of peptide modification.