

The Protein Facility of the Iowa State University Office of Biotechnology

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The Protein Facility of the Iowa State University Office of Biotechnology is open to faculty and students from the university, other educational institutions, and industry scientists. The facility offers the following services:

- Protein/peptide sequencing
- Large and small scale peptide synthesis (Fmoc)
- Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry
- Circular dichroism
- SDS-PAGE/blotting
- 2-D gel electrophoresis
- Isoelectric focusing (IEF)
- In-gel digestion/peptide mass fingerprinting
- Digital image acquisition and analysis using the Typhoon imaging system and the gel documentation system
- 1D and 2D gel analysis software
- Preparative, analytical and micro-analytical high performance liquid chromatography (HPLC)

Other services are also available upon request.

Personnel:

Amanda Brockman

Margie Carter

Joel Nott, manager

Protein/Peptide Sequencing

The chemical procedure employed by automated protein/peptide sequencers is derived from the method originated by Pehr Edman in the 1950s for the sequential degradation of peptide chains. Phenylisothiocyanate (PITC) reacts with the amino acid at the amino terminus under basic conditions (provided by N-methylpiperidine/methanol/water) to form a phenylthiocarbamyl derivative (PTC-protein). Trifluoroacetic acid (TFA) then cleaves off the first N-terminal amino acid as an anilinothiazolinone derivative (ATZ-amino acid) and leaves the new N-terminus for the next degradation cycle. The ATZ-amino acid is then removed by extraction with N-butyl chloride and converted to a phenylthiohydantoin derivative (PTH-amino acid) with 25% TFA/water (see Figure 1). Several by-products are also formed during the Edman degradation chemistry and are shown in Figure 2. The PTH-amino acid is then transferred to a reverse-phase C18 column for detection at 270 nm. This process is repeated sequentially to provide the N-terminal sequence of the protein/peptide. A standard mixture of 19 PTH-amino acids is also injected onto the column for separation (usually done as the first cycle of the sequencing run). This chromatogram provides standard retention times of the amino acids for comparison with each Edman degradation cycle chromatogram. The HPLC chromatograms are collected using a computer data analysis system. To determine the amino acid present at a particular residue number, the chromatogram from the residue of interest is compared with the chromatogram from the previous residue by overlaying one on top of the other. From this, the amino acid for this particular residue can be determined (see Figure 3).

Sample Preparation for Protein Sequencing

Sample Amount	10-100 pmols is preferred, although a lower amount is acceptable.
Sample Form	I. In 30-150 microliters of volatile solvents such as water, acetonitrile, propanol, acetic acid, or formic acid. II. On a PVDF membrane. A sample should be as concentrated as possible on the PVDF membrane (e.g. 1 µg/lane). Several bands can be used. The bands should be stained with Coomassie Blue, Ponceau S, or Amido Black. After staining/destaining, a blotted membrane must be rinsed thoroughly with deionized water. The whole membrane may be submitted with the bands marked or the bands may be cut out and submitted.
Purity	For liquid samples, the solution should contain only one protein or peptide. Buffers, SDS, salts, amino acids, primary amines, and other contaminants must be removed from your sample. These contaminants may affect the Edman degradation reaction on the instrument, contaminate the instrument or affect PTH amino acid detection. Samples submitted on PVDF (blotted from SDS-PAGE gels) should have well separated bands to minimize contamination.

**Cysteine
Modification**

Since unmodified Cys residue cannot be detected, Cys should be modified (according to Fulmer, C.S. (1984) Anal. Biochem. **142:** 336) before sample submission if you wish to identify Cys.

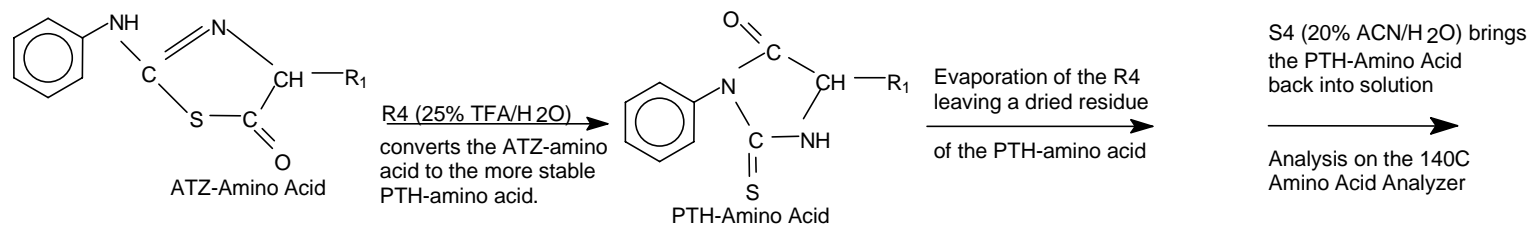
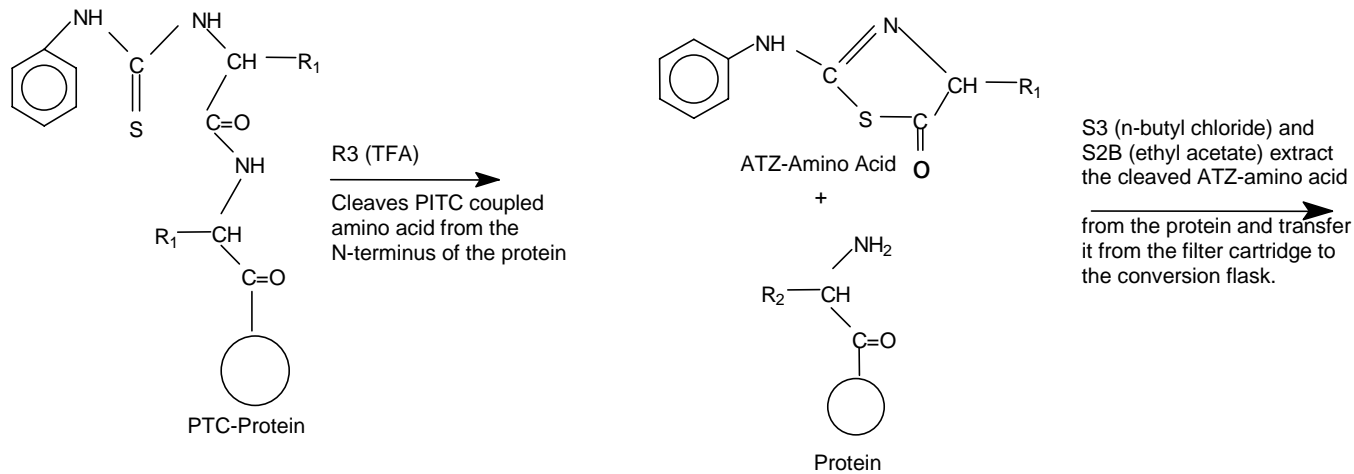
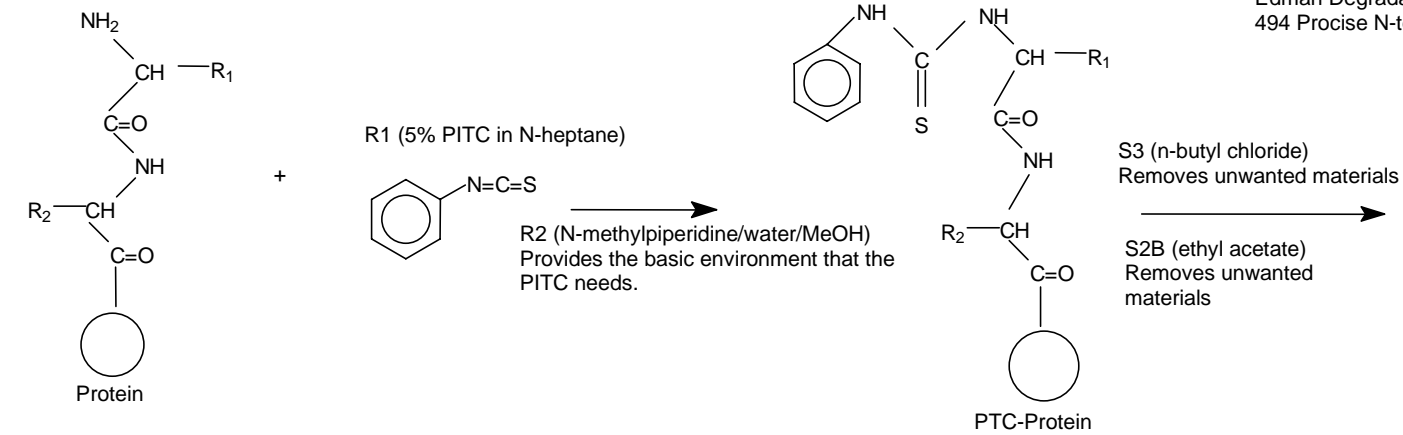
**N-terminal
Blockage**

If the amino terminus is blocked, the protein or peptide cannot be sequenced using Edman degradation. We perform de-blocking procedures and will discuss options with you if blockage is suspected.

**Glycosylation and
Other Modifications**

Glycosylated amino acids and phosphorylated amino acids may result in blank cycles, reduced peaks or altered retention times.

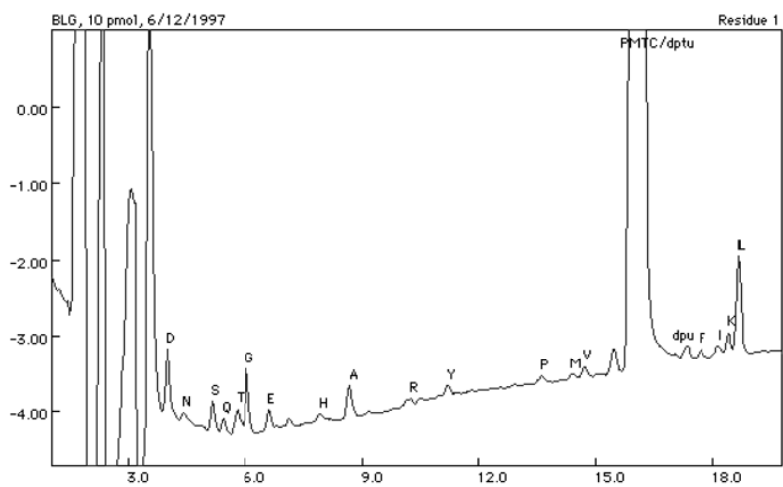
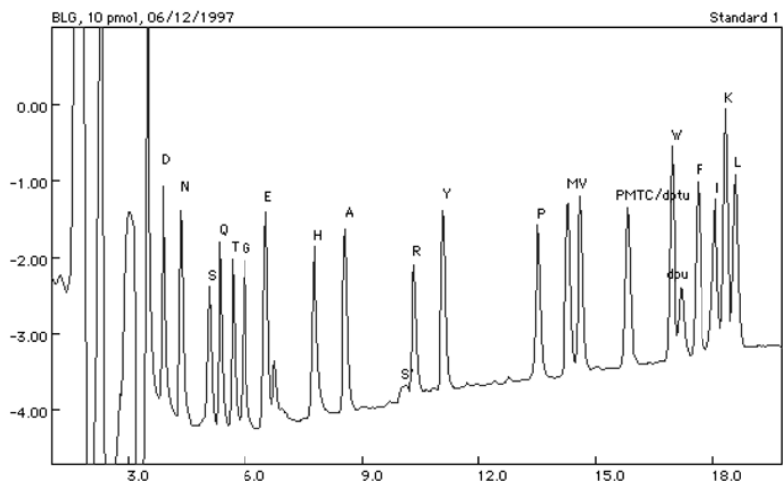
Edman Degradation on the
494 Procise N-terminal Sequencer



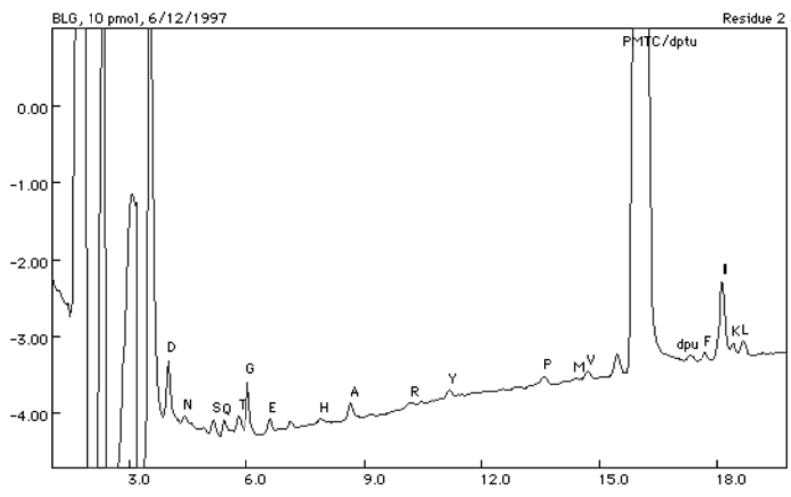
PITC - Phenylisothiocyanate
PTC - Phenylthiocarbonyl

TFA - Trifluoroacetic acid
PTH - Phenylthiohydantoin

ATZ - Anilinothiazolinone



L



I

Column: C-18, 2.1 mm X 220 mm
Solvent: A: 3.5% Tetrahydrofuran/water
 Buffer Premix added
 .005% TFA
 B: Acetonitrile/2-Propanol, 8-90%
Flow: 325 uL/minute
Detection: 269 nm

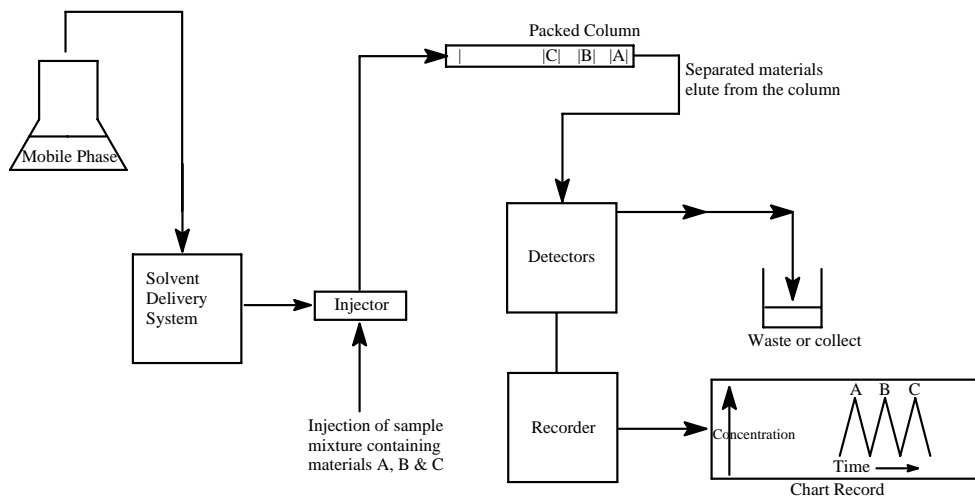
Liquid Chromatography

Introduction--High-performance-liquid chromatography (HPLC) is at present the most common technique used to purify and characterize peptides and proteins. Used in conjunction with gel electrophoresis, it offers to the protein chemist an extremely powerful tool which has enabled the design of purification protocols unrealized before its advent. It is now possible to purify and characterize minute amounts of protein and peptides from a vast array of starting material from contaminating material. The general proliferation of HPLC-based techniques can be directly related to such attributes as sensitivity, speed, cost, and convenience.

Chromatography is a method of separating chemical mixtures into individual components. It is a separation technique, not an identification technique. In contrast, spectrophotometry can identify the presence of specific molecules in a sample mixture, but its ability to provide accurate quantitative information is limited when the sample mixture is composed of components of similar chemical structure, as it cannot separate the components. For this reason, liquid chromatography is very often coupled with spectrophotometry including ultraviolet and visible absorption, laser excitation and fluorescence methods. For example, chromatography provides the most practical method of identifying and quantifying the individual B vitamins from a multi-vitamin tablet. The same situation applies in the analysis of polymers and many other types of organic compounds.

The components of a liquid chromatography system are shown in the figure below.

Block diagram showing the components of an HPLC instrument



With liquid chromatography the sample mixture to be analyzed is dissolved in a suitable solvent (ideally this solvent is identical to the mobile phase) and introduced, by means of an injection device, onto the head of the column. The sample is then carried through the column by a continuous flow of mobile phase from the pump.

Some components of the sample will travel through the column more slowly than others. Those components that are more attracted to the column packing material are thus separated by the time

they emerge, or elute, from the column end. Typically, an ultraviolet absorbance or refractive index detector monitors the components as they emerge from the column through a beam of light located at the end of the column. The detector transmits signals to a recording device that typically plots the data on a chart. This chromatogram may provide both qualitative and quantitative information about the sample.

Liquid chromatography is an innovation of analytical chemistry for both chemists and biologists. Below is a list of some of the application areas where liquid chromatography is a vital analytical technique.

Applications Areas

1. Pharmaceuticals
 Research & Quality Control
2. Clinical
 Drug monitoring, Disease Markers
3. Forensics
4. Pesticides
 Research & Quality Control
 Environmental Analysis
5. Biochemical Research
6. Food & Beverage
7. Polymer/Plastics

There are four major modes of liquid chromatographic separation:

- Size exclusion chromatography
- Normal phase chromatography
- Ion-exchange chromatography
- Reversed phase chromatography

Size Exclusion Chromatography--Size exclusion chromatography (SEC) is a general term which includes both GPC (Gel Permeation Chromatography) and GFC (Gel Filtration Chromatography). GPC is considered organic-based SEC and GFC is aqueous-based SEC.

SEC presents the simplest separation mechanism in chromatography. Optimally there is no adsorption involved, and the mobile phase should be considered as a carrier phase, not one which has a large effect on the chromatography. Molecules are actually separated on the basis of molecular size in solution. This is achieved with a porous packing material which is compatible with the mobile phase. The smallest components in the sample migrate into the smallest pores of the packing while the molecular dimensions of the higher-molecular-weight components prevent them from penetrating as far into the pores. Therefore the largest molecules will elute first and the smallest molecules will elute last.

SEC is more susceptible to flow than any other chromatography. This is due to
1) HETP (Height equivalent to a theoretical plate) will increase rapidly with flow.

- 2) High flow rates simply push the molecules through the column, not allowing complete permeation of the pore volumes.
- 3) Calibrations and subsequent calculations are based on flow; reproducible, accurate flow is crucial for accurate results.

Normal Phase Chromatography

1. Adsorption Chromatography

This is the oldest form of liquid chromatography. The technique depends upon specific interactions of the solute with the surface of a finely divided adsorbent. The adsorbent can be silica gel (the most popular), alumina, or charcoal (rarely used). Generally, we understand that the mobile phase and the solute molecules are in competition for the active sites of the adsorbent. The adsorbent-solute interaction is the most important parameter governing the separation by adsorption chromatography. If the solute has a higher affinity for the mobile phase, the compound will not be strongly retained. If the solute has a higher affinity for the stationary phase, it will be strongly retained. In other words, the higher the polarity, the stronger the interaction; thus, the longer the retention time.

2. Bonded Normal Phase Chromatography

This is similar to silica, but the silanols are chemically bonded with a polar stationary phase. The basic mechanism is based on the partitioning of the solute between the mobile phase and the stationary phase. Very similar to extraction techniques.

Ion-Exchange Chromatography--Ion exchange separations are based upon attractive ionic forces between molecules carrying charged groups of opposite charge to the charge on the stationary phase. Separations are made between a polar mobile liquid (usually water) containing salts or small amount of alcohols, and a stationary phase containing either acidic or basic fixed sites. The separation depends upon the ionic nature of the compound (e.g. pKa), the polarizability of the molecule, the solvation shell of the molecule, and the relative attraction of the compound for the ion exchange surface. After the sorption of the charged sample, desorption is brought about by increasing the salt concentration (ionic strength) in the mobile phase or by changing the pH of the mobile phase. Both ionic strength and pH can minimize the charge (electrostatic) attraction between the sample and the stationary phase, and hence, make the mobile phase stronger.

Ion-exchange chromatography separation results when sample components move through the column at different speeds. At low ionic strengths, all components with an electrostatic attraction for the ion exchanger will be tightly held on top of the column. When the ionic strength of the mobile phase is increased by adding a salt, the salt ions compete with the adsorbed sample ions for the bonded charges on the column. As a result, some of the sample components will be partially desorbed and start moving through the column. If the salt concentration is higher, the resulting ionic strength causes a larger number of the sample components to be desorbed, and the speed of the movement down the column increases. The stronger the charge attraction of the sample to the column is, the higher the ionic strength needed to bring about desorption.

At a certain level of ionic strength, no sample components are held by the charges bonded to the column. In this case, all sample components will elute at the mobile phase volume (V_0). Somewhere in between total adsorption and total desorption one will find the optimal selectivity for a given pH value of the mobile phase. Thus to optimize retention in ion-exchange chromatography, a pH value is chosen that creates sufficient charge differences among the sample components. Then, an ionic strength is selected that competes with these charge differences so that the relative movement of each component through the column results in the desired selectivity.

Reversed Phase Chromatography--In reversed phase chromatography, the packing is nonpolar and the solvent is polar with respect to the sample. Retention is the result of the interaction of the nonpolar components of the solutes and the nonpolar stationary phase. Typical stationary phases are nonpolar hydrocarbons, waxy liquids or bonded hydrocarbons (such as C_{18} , C_8 , C_4 , etc.) and the solvents are polar aqueous-organic mixtures such as methanol-water or acetonitrile-water.

The C_{18} , C_8 , and phenyl bonded phases are most often used in the reverse phase mode. It has been estimated that 60-90% of all analytical LC separations are done on bonded phases in the reversed phase mode. Bonded phases made by covalently bonding a molecule onto a solid stationary phase are intended to prepare "liquid coatings" which will be permanent. Silica is a reactive substrate to which various functionalities can be attached or bonded. The functionalities most widely bonded to silica are the alkyl (C_{18} and C_8), aromatic phenyl, and cyano and amino groups.

General characteristics of reversed phase chromatography

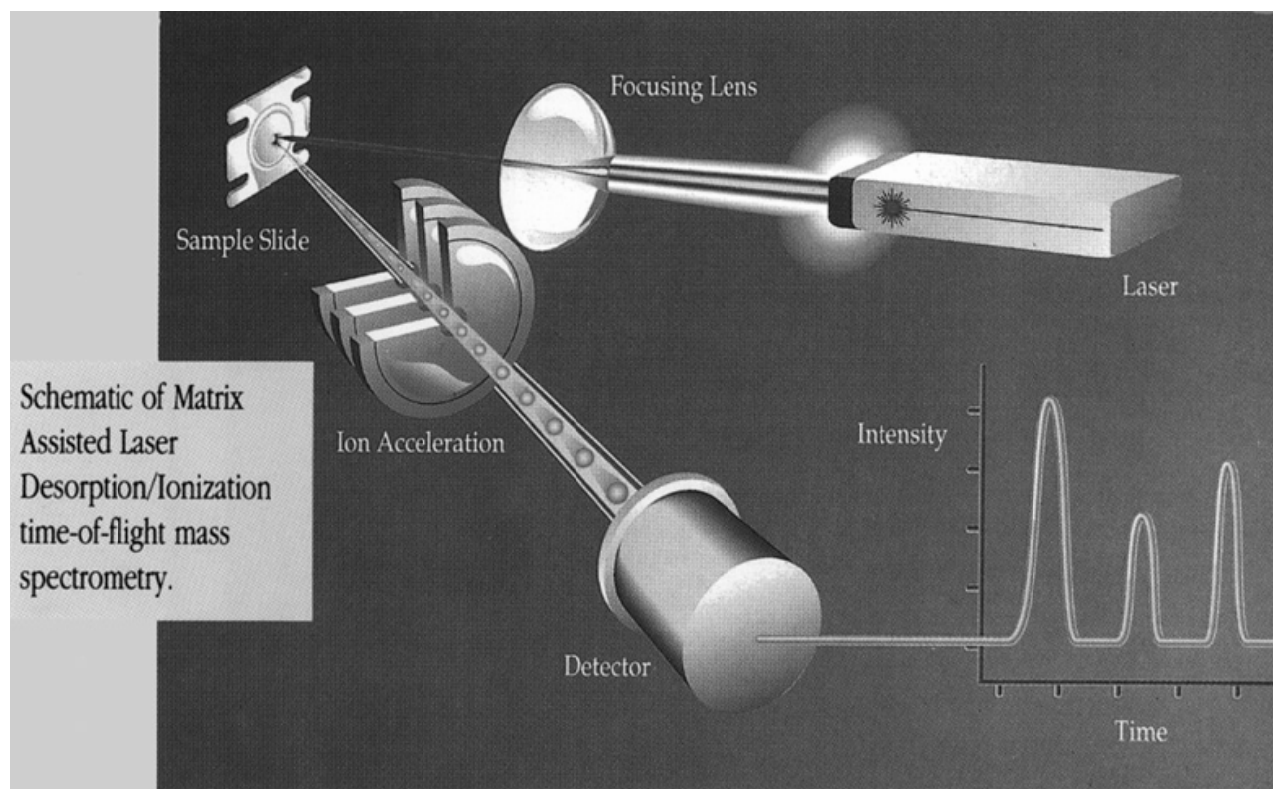
- Broad scope which allows sample types with a wide range of polarities and molecular weights to be separated.
- General rapidity of mobile phase column equilibration during methods development and gradient regeneration.
- General ease of use.
- Applicability to separation of ionic or ionizable compounds by manipulating secondary chemical equilibrium such as ionization control and ion pairing in the aqueous mobile phase.
 - ◆ Buffering the mobile phase in the pH range from 2 to 5 with one of the common buffers, the ionization of the weak acids can be suppressed or controlled allowing them to be retained in their neutral form. Similarly weak bases can be retained in their neutral form at pH 7-7.5.
 - ◆ For strong acids and bases ionization control cannot be employed because the stability of alkyl bonded phases is diminished below pH 2 and above pH 7.5. Highly hydrophilic weak acids and bases often remain difficult to retain with ionization control. In such cases ion pair reversed phase chromatography can be used. In this method, counterions (species of opposite charge to the solutes) thereby regulate the retention. Typically alkyl amines or tetra alkyl amines are added to ion pair with acids whereas alkyl sulfates, sulfonates, or phosphates are

used to ion pair with bases. The technique is an alternative to ion exchange chromatography for analysis of ionic compounds.

- The possibility of special selectivity such as structural or steric are achievable by specific mobile phase additives:
 - ◆ Metal ions are capable of binding to organic compounds in a very selective method which is used for ligand exchange chromatography. The selectivity generated in these metal ion phase systems is based in part on differences of the solute (ligand) binding strength to the metal ion. An alternate approach is the addition of various chelating agents (4-dodecyldiethylene-triamine- C_{12} dien) in combination with a metal ion. The type and strength of the metal chelate complex-solute binding can be greatly varied depending upon the chemical environment surrounding the metal ion as determined by the chelating agent added.

MALDI Mass Analysis

Matrix Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) is a mass analysis technique that was pioneered by Professor Franz Hillenkamp and Dr. Michael Karas of the University of Münster in Germany. The facility has a PerSeptive Biosystems Voyager-DE PRO. MALDI offers a quick and easy method of mass analysis using a minimal amount of sample. The principles involved in the MALDI mass analysis can be seen in the figure below and are described following the figure.



From Finnigan MAT

Principles of MALDI

- The sample is dispersed in a large excess of matrix material which will strongly absorb the incident light.
 - ◆ The matrix contains chromophore for the laser light and since the matrix is in a large molar excess it will absorb essentially all of the laser radiation
 - ◆ The matrix isolates sample molecules in a chemical environment which enhances the probability of ionization without fragmentation
- Short pulses of laser light focused on to the sample spot cause the sample and matrix to volatilize
- The ions formed are accelerated by a high voltage supply and then allowed to drift down a flight tube where they separate according to mass
- Arrival at the end of the flight tube is detected and recorded by a high speed recording device

The time-of-flight of the ion is converted to mass using the following principles:

- An accelerating potential (V) will give an ion of charge z an energy of zV. This can be equated to the kinetic energy of motion and the mass (m) and the velocity (v) of the ion
$$zV = 1/2mv^2$$
- Since velocity is length (L) divided by time (t) then
$$m/Z = [2Vt^2]/L^2$$
- V and L cannot be measured with sufficient accuracy but the equation can be rewritten
$$m/Z = B(t-A)^2$$
where A and B are calibration constants that can be determined by calibrating to a known m/Z

Mass of an ion on the Dynamo is determined by the following method

1. Measure time-of-flight (t) of the ion
2. External or internal calibration is used to determine the constants A and B so the time-of-flight can be converted to mass
$$m/Z = B(t-A)^2$$
3. Store B/V so changes in the 20 kV voltage supply does not effect calibration
It is assumed that all ions have the same kinetic energy.

Samples are loaded onto metal plates for analysis on the instrument. A sample concentration of 1 mg/mL is ideal and usually from one to ten picomoles of sample is required for analysis. This is spotted onto the sample position on the metal strip and then 0.5 μ L of matrix (usually 10 mg/mL) is applied to the sample position as well. There are many different matrices that can be used for MALDI-TOF. Some of the most common include Sinapinnic Acid (SA) for protein samples, α -Cyano-4-hydroxycinnamic acid (ACH) for peptide samples, and a 9:1 mixture of 2,5-Dihydroxybenzoic acid and 2-hydroxy-5-methoxybenzoic acid (sDHB) for carbohydrate and sometimes protein samples. DNA can also be analyzed using MALDI-TOF by employing different matrices. New matrix solutions are now in development which will yield greater sensitivity and resolution.

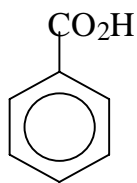
MALDI technology has many applications in the biochemical field. It can be used to easily monitor and optimize enzymatic digests, characterize proteins, or can be used for quality control for peptide synthesis. MALDI has also been used as a method of N-terminal and C-terminal protein/peptide sequencing. There are also applications in the rapid conformation of post translational modifications and the quantitation of drugs and chelators conjugated to monoclonal antibodies.

MALDI Sample Preparation

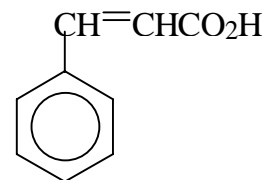
MALDI samples should be free of SDS, and should not be radioactive. It is best to remove buffer salts and detergents (e.g. by dialysis) prior to analysis and to dissolve the sample in a suitable solvent (e.g. 0.1% TFA/water) which will not degrade the spectrum. If there is too much salt in a sample, the salt signal intensity is so large that it effectively suppresses out the sample signal, giving no sample spectrum. In cases where it is not possible to remove these contaminants the sample should be in a higher concentration. It may then be possible to dilute the sample to the point where the contaminants will have little effect on the spectrum. Levels of buffers and detergents which exceed the following limits will probably cause noticeable degradation of the spectrum:

Phosphate buffer	>50mM
Ammonium bicarbonate	>30mM
Tris buffer	>100mM
Guanidine	>1M
Detergents(e.g. Triton-X)	>0.1%
SDS	>0.01%
Alkali metal salts	>1M
Glycerol	>1%
Sodium Azide	>1mM

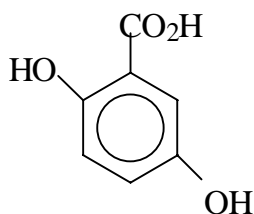
Common matrices used for MALDI



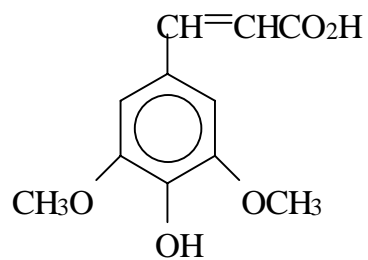
Benzoic acid



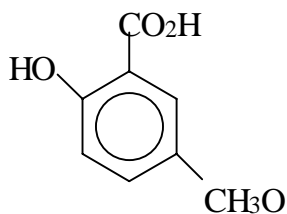
Cinnamic Acid



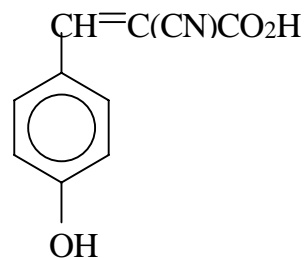
2,5-Dihydroxybenzoic acid
(DHB)



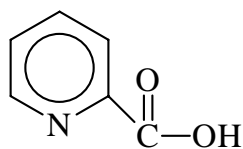
3,5-Dimethoxy-4-hydroxy-cinnamic acid
(Sinapinic Acid)



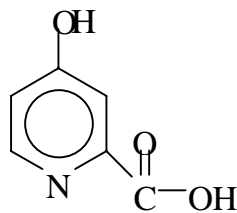
2-hydroxy-5-methoxybenzoic acid
(2H5MBA)



α -Cyano-4-hydroxycinnamic acid
(ACH)



Picolinic acid



3-Hydroxypicolinic acid
(3HPA)

Solid Phase Peptide Synthesis

Solid phase peptide synthesis developed by R. B. Merrifield was a major breakthrough allowing for the chemical synthesis of peptides and small proteins. The general scheme of solid phase peptide synthesis is illustrated in Figure 1. An insoluble polymer support (resin) is used to anchor the peptide chain as each additional α -amino acid is attached. This polymer support is constructed of 20-50 μm diameter particles which are chemically inert to the reagents and solvents used in solid phase peptide synthesis. These particles swell extensively in solvents, which makes the linker arms more accessible.

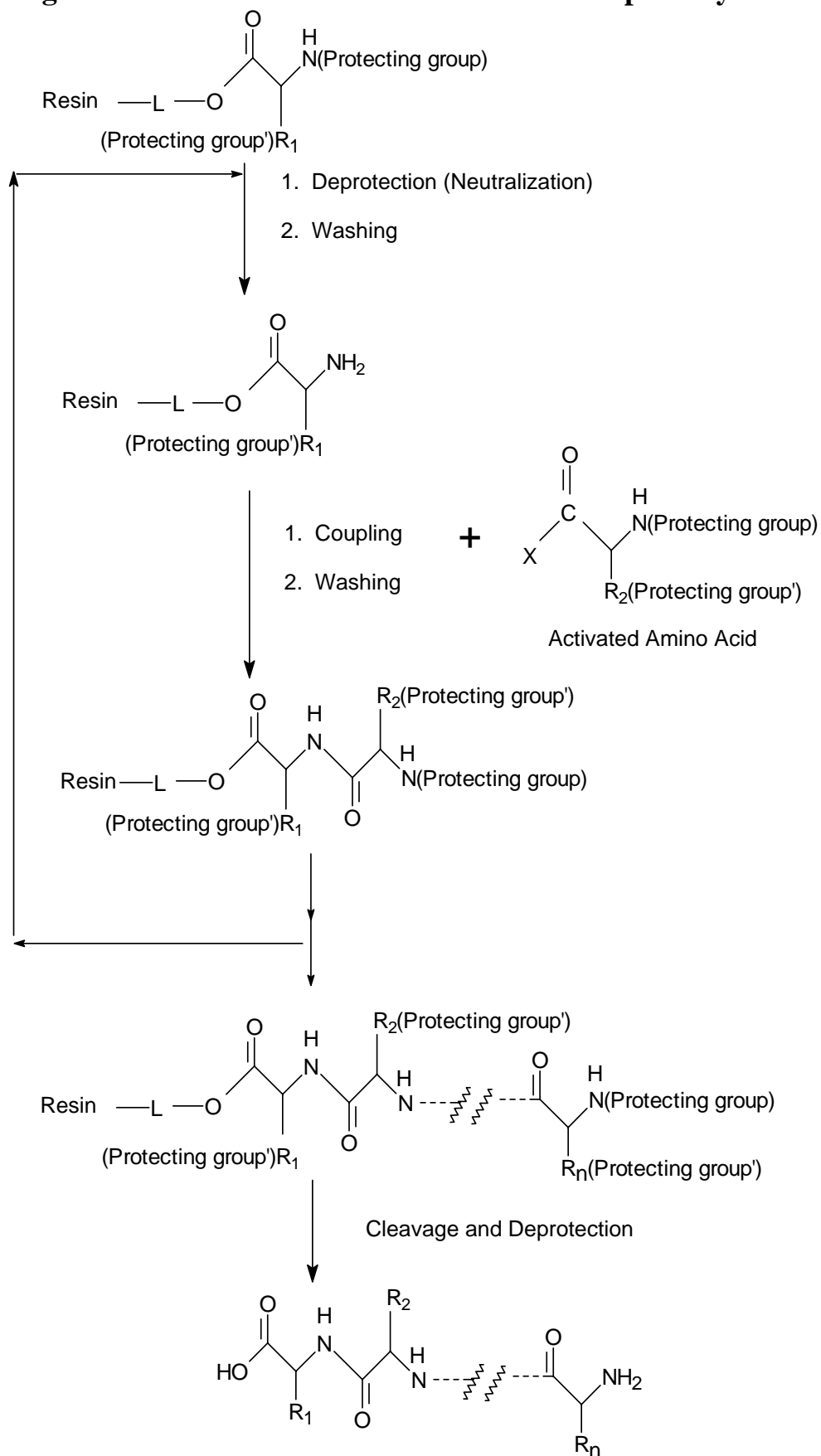
Organic linkers attached to the polymer support activate the resin sites and strengthen the bond between the α -amino acid and the polymer support. Chloromethyl linkers, which were developed first, have been found to be unsatisfactory for longer peptides due to a decrease in step yields. The PAM (phenylacetamidomethyl) resin, because of the electron withdrawing power of the acid amide group on the phenylene ring, provides a much more stable bond than the classical resin. Another alternative resin for peptides under typical peptide synthesis conditions is the Wang resin. This resin is generally used with the Fmoc labile protecting group.

A labile group protects the α -amino group of the amino acid. This group should be easily removed after each coupling reaction so that the next α -amino protected amino acid may be added. Typical labile protecting groups include t-Boc (*tert*-butyloxycarbonyl) and Fmoc (9-fluorenylmethyloxycarbonyl). t-Boc is a very satisfactory labile group which is stable at room temperature and easily removed with dilute solutions of trifluoroacetic acid (TFA) and dichloromethane. Fmoc is a base labile protecting group which is easily removed by concentrated solutions of amines (usually 20-55% piperidine in *N*-methylpyrrolidone). When using Fmoc α -amino acids, an acid labile (or base stable) resin, such as an ether resin, is desired.

The stable blocking group protects the reactive functional group of an amino acid and prevents formation of complicated secondary chains. This blocking group must remain attached throughout the synthesis and may be removed after completion of synthesis. When choosing a stable blocking group, the labile protecting group and the cleavage procedure to be used should be considered.

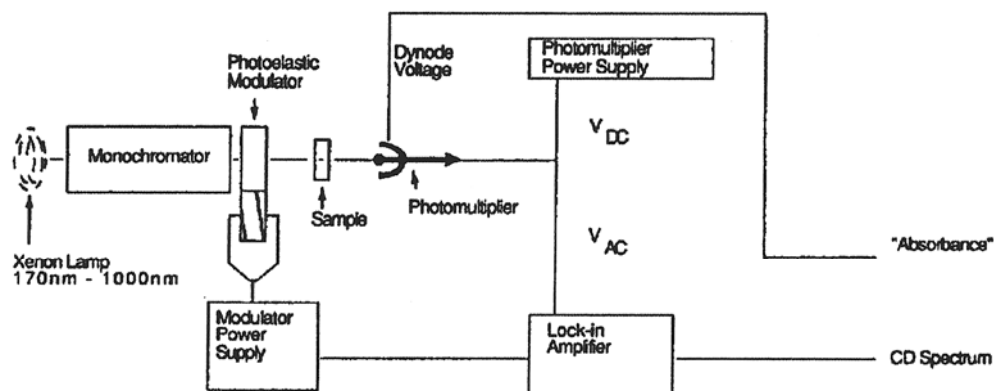
After generation of the resin bound synthetic peptide, the stable blocking groups are removed and the peptide is cleaved from the resin to produce a "free" peptide. In general, the stable blocking groups and organic linkers are labile to strong acids such as trifluoroacetic acid (TFA). After the peptide is cleaved from the resin, the resin is washed away and the peptide is extracted with ether to remove unwanted materials such as the scavengers used in the cleavage reaction. The peptide is then frozen and lyophilized to produce the solid peptide. This is then characterized by HPLC and MALDI before being used by the investigator. It may also be necessary to purify the peptide by HPLC if higher purity is required by the investigator.

Figure 1: General Protocol for Solid Phase Peptide Synthesis



Circular Dichroism Spectroscopy

Circular dichroism (CD) spectroscopy is an optical technique that allows the detection and quantitation of the chirality of molecular structures. It also provides information about the secondary and tertiary structures of proteins. Circular dichroism is the difference in the absorption of left and right circularly polarized light. A periodic variation in the polarization of the light beam is induced by the polarization modulator through all ellipticities from left circular through elliptical, unchanged linear and elliptical to right circular. This polarized light passes through the sample to a photomultiplier detector. If the sample is not optically active, the light beam does not vary through this cycle. With the introduction of an optically active sample, a preferential absorption is seen during one of the polarization periods and the intensity of the transmitted light now varies during the modulation cycle. The variation is directly related to the circular dichroism of the sample at that wavelength. Successive detection is performed at various wavelengths leads to the generation of the full CD spectrum. For CD to be exhibited for a sample, the sample must be optically active and not superposable on its mirror image.



The CD spectrometer.

Information CD can provide

- Protein secondary structure
Used to estimate the secondary structure (conformation) of the polypeptide chain (α -helix, β -sheet)
- Nucleic acid conformation
Enhanced CD that is representative of the relative base-base orientation
- Interaction studies
Direct, noninvasive means of monitoring binding of molecules to proteins or enzymes

From *The Protein Protocols CD-ROM*, J.M. Walker, ed., Humana Press, Totowa, NJ, 1998

SDS-PAGE/Electroblotting

We currently use a Mini-PROTEAN II Dual Slab Cell system and a Mini Trans-Blot Electrophoretic Transfer Cell system, both from Bio-Rad Laboratories. The following is a general description of SDS-PAGE and blotting for amino acid analysis and N-terminal protein sequencing.

SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in the presence of a reducing agent (2-mercaptoethanol) is a technique for the separation of polypeptide subunits according to their molecular weight. The protocol involves denaturing the protein sample by heating it in the presence of SDS and a reducing agent. SDS will bind to the protein causing it to unfold, whereas the reducing agent will reduce the intramolecular and intermolecular disulfide bonds. The binding of SDS by the protein confers a net negative charge and the denatured polypeptide will migrate through a gel of known percent acrylamide in the presence of an applied electric field towards the positive electrode (anode). After the electrophoresis is complete, the gel is stained with Coomassie Blue R-250 to visualize the polypeptide bands. The molecular weight of the polypeptide is inversely proportional to its mobility. The molecular weight of the polypeptide subunit can be estimated directly from a semilog graph of the molecular weight of standard proteins versus their mobility or from a plot of the log of molecular weight versus mobility. Separation of proteins by SDS-PAGE is an excellent technique for producing individually "purified" proteins.

Electroblotting

Blotting is a technique for the electrophoretic transfer of DNA, RNA or protein to a suitable membrane. The method most commonly used for the electrotransfer of proteins to nitrocellulose is that reported by Towbin et al. (1979). In order to take advantage of this technique for the purpose of amino acid analysis or N-terminal sequencing, the proteins must be transferred to a membrane that is stable to the chemicals used in these analytical procedures. For protein sequencing and amino acid analysis the proteins are transferred to a chemically stable membrane, polyvinylidene difluoride (PVDF). In our laboratory we use the "wet" transfer technique, rather than the "dry" transfer technique. Proteins are first separated by SDS-PAGE, the gel is removed from the electrophoresis cassette (do not stain the gel before blotting) and equilibrated in transfer buffer without methanol. The PVDF membrane is "activated" by dipping it in methanol; it is then placed in transfer buffer containing methanol. The gel-PVDF sandwich is placed in a specially designed holder that in turn is placed in the buffer-containing electrophoresis unit. At the pH of the buffer (pH 8.3) most proteins are negatively charged and will migrate to the anode (positive electrode). In case one suspects the protein has a pI greater than 8.3, a PVDF membrane can be placed at the cathode-side of the gel as well. Alternatively, the pH of the transfer buffer can be adjusted to a higher pH. After transfer, the membrane is stained with Coomassie Blue R-250 and destained to locate the protein bands. Sections containing the protein bands can then be excised for amino acid analysis and/or N-terminal protein sequencing.

Isoelectric Focusing (IEF)

IEF is an electrophoretic method for separating proteins based on their isoelectric point. The isoelectric point is the pH at which the net charge of the protein is zero. With the presence of a pH gradient in the IEF technique, the protein will migrate to the position in the gradient where its charge is zero. Proteins with a positive net charge will migrate toward the cathode until it meets its pI. Proteins with a negative net charge will migrate toward the anode until it meets its pI. If the protein diffuses away from its pI, it will regain its charge and migrate back. This focusing effect allows proteins to be separated based on very small charge differences. IEF is performed under high voltages (> 1000 V) until the proteins have reached their final position in the pH gradient. If IEF is performed under denaturing conditions very high resolution and cleanliness of sample can be obtained.

IEF is performed using the IPGphor Isoelectric Focusing System from Amersham Pharmacia Biotech. This system uses immobilized pH gradient (IPG) gels with a plastic backer. These gels increase reproducibility, loading capacity and are easier to handle. The IPG strips are available in either 7cm (for mini-gel application) or 18cm (for larger gel applications) sizes.

2-D Gel Electrophoresis

The technique of two-dimensional electrophoresis involves separating proteins in the first dimension according to charge (isoelectric focusing, IEF), followed by separating the focused proteins in the second dimension according to molecular weight by sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE). The proteins may be visualized by staining with Coomassie Brilliant Blue R250, silver stain, fluorescent dyes or through radioisotope detection (phosphorescence) after the proteins are metabolically labeled with ^3H , ^{14}C , or ^{35}S -labeled amino acids. This two-dimensional array will produce spots that correspond to a single protein species in the sample. Using this technique combined with the 2D gel analysis system, thousands of different protein can be separated and information such as pI, molecular weight and protein amount can be determined. These spots can be excised for further analysis or the 2-D array can be analyzed for differences in protein quantity or in proteins present in the gel. 2-D gels can also be electroblotted to PVDF or nitrocellulose membranes for further analysis.

2-D electrophoresis is performed using the IPGPhor system for the 1st dimension separation, followed by 2nd dimension separation using either a Bio-Rad Mini-Protean II system or the Amersham Pharmacia Biotech Hoefer DALT system (20 cm x 23 cm gels).

In-gel digestion/peptide mass fingerprinting

The Investigator ProGest from Genomic Solutions is used for the automated in-gel digestion of protein samples from 1-D or 2-D gels. Gel spots can be digested with a variety of enzymes including trypsin, Arg-C and Glu-C. The resulting peptides from the digestion can then be analyzed by MALDI-TOF. From the spectrum obtained, peptide mass fingerprinting (using databases available on the WWW) can be performed to identify the original protein. The peptides from the digested proteins can also be separated by HPLC for further analysis by N-terminal sequencing or MALDI-TOF.

Digital image acquisition and analysis using the Typhoon imaging system and the 2D gel documentation/analysis system

The facility has two methods for the acquisition of digital gel images. For the acquisition of Coomassie Blue stained gels, the Amersham Pharmacia Biotech ImageScanner flatbed scanner with transparency module can be used. Once the gel image has been digitized, 2-D gel analysis can be performed using Progenesis SameSpots from Nonlinear Dynamics. This software allows the comparison of spots within 1 or more gels for differences in quantity. Gels can also be compared for the presence or absence of proteins of interest. This can be useful in determining protein spots that may be candidates for further analysis.

The Typhoon 8600 from Amersham Pharmacia Biotech is a multimode image scanner that can be used for the acquisition of phosphorescence, fluorescence or chemiluminescent samples. The scanner is capable of imaging gel sandwiches, agarose and polyacrylamide gels, membranes, microplates and microarrays. Samples can also be labeled with multiple dyes and imaged on the Typhoon. After image acquisition, the image can be analyzed for quantitative data using ImageQuant software from Amersham Pharmacia Biotech.