Commonly Used Analytical Techniques for Biotechnology Products

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ANALYTICAL TOOLS FOR PROTEINS

Characterization of proteins and peptides has long been interesting to pharmaceutical scientists. As the progress of the Human Genome Project reveals the human genome sequence, the functional research of discovered genes has moved forward to the systematic analysis of the components that constitute biologic systems. And the most important components relevant to the biological activity of the cells are proteins. Since many biological reactions are greatly affected by proteins such as enzymes, receptors, and intrinsic or extrinsic proteins, the structural analysis of these proteins and the relationship of the structure and functionality are very important in peptide protein research. Several analytical techniques such as amino acid sequencing, x-ray diffraction, x-ray crystallography, circular dichroism (CD), Fourier transform infrared spectroscopy (FTIR), electrophoresis, and mass spectrometry (MS) have been used for this purpose. This chapter describes the methods of detection mentioned above and delves into only a fraction of the applications and variations of these techniques.

SPECTROSCOPIC AND PHOTOMETRIC METHODS

ULTRAVIOLET-VISIBLE (UV-VIS)

UV-VIS is one of the spectrophotometric methods that has been extensively used for the identification and quantitative analysis of proteins (Wetlaufer 1962). Spectrophotometry, in general, takes advantage of the quantum mechanical nature of chemical compounds to absorb, scatter, or modify the properties of a beam of electromagnetic radiation (photons) or induce changes in the molecular properties. The UV-VIS technique uses photons in the ultraviolet to visible regions, with wavelengths between 190 nm and 900 nm. Research has shown that proteins contain several chromophores that absorb light in the ultraviolet and infrared regions. Many of them also display fluorescence. The most important chromophores are the aromatic rings of Phe, Tyr, and Trp (Wang 1992). UV absorbance and fluorescence are useful probes of structure and structural changes of proteins. This is due to the fact that chromophores display shifted spectra upon increasing or decreasing polarity of their environment, with changes in wavelength of maximum absorbance (lambda max) and molar extinction coefficient possible. A relation between the level of absorption (absorbance) and beam wavelength can be determined. These spectra are unique for each molecule, since each molecule is unique in quantum structure.

Proteins in solution absorb ultraviolet light with absorbance max at 280 and 200 nm (Wang 1992). Amino acids with aromatic rings are the primary reason for the absorbance peak at 280 nm. Peptide bonds are primarily responsible for the peak at
200 nm. Secondary, tertiary, and quaternary structure all affect absorbance, therefore factors such as pH, ionic strength, and so forth can alter the absorbance spectrum.

Several types of spectrophotometers are available for the UV-VIS range of wavelengths, all of which are single beam- or double beam type-instruments. A single beam spectrophotometer, as the name implies, uses a single beam to adjust the transmittance of a blank solution to 100%. This calibration is exercised prior to measurement at each wavelength. A transmittance is a measure of photonic transmission. At 100% transmittance, the molecule absorbs and then de-excites to release the photon. A more commonly used spectrophotometer is the double beam type. This type of detector uses two beams from a single photon source. One beam passes through a reference cell, and another the sample cell. Measurements of absorbance are taken simultaneously for both cells, eliminating the readjustment of the transmittance at each wavelength. The recording of spectral scans is also simplified (Wetlaufer 1962; Wang 1992).

Some colored compounds have also been linked to peptides or proteins for the detection and measurement within visible spectra ranges. Colorimetric methods are relatively nonspecific but they can be made specific by linking them to enzymatic or immunologic reactions (see Immunoassay section). In these cases, enzymes produce colored products from colorless substrates, rather than radionuclides, are used to label particular antigens and antibodies (Winder and Gent 1971).

**INFRARED SPECTROSCOPY**

Infrared spectroscopy measures the frequency or wavelength of light absorbed by the molecules caused by transitions in vibrational energy levels (Vollhardt and Schore 1995). The wavelengths of IR absorption bands are characteristic of specific types of chemical bonds. In the past infrared had little application in protein analysis due to instrumentation and interpretation limitations. The development of Fourier transform infrared spectrophotometry (FTIR) makes it possible to characterize proteins using IR techniques (Surewicz et al. 1993). Several IR absorption regions are important for protein analysis. The amide I groups in proteins have a vibration absorption frequency of 1630–1670 cm\(^{-1}\). Secondary structures of proteins such as alpha(\(\alpha\))-helix and beta(\(\beta\))-sheet have amide absorptions of 1645–1660 cm\(^{-1}\) and 1665–1680 cm\(^{-1}\), respectively. Random coil has absorptions in the range of 1660–1670 cm\(^{-1}\). These characterization criteria come from studies of model polypeptides with known secondary structures. Thus, FTIR is useful in conformational analysis of peptides and proteins (Arrondo et al. 1993).

Through the advanced development of IR instruments, measurements can also be performed in aqueous solutions, organic solvents, detergent micelles as well as in phospholipid membranes. In a study carried out by Vigano et al. (2000), proteins in live cells were characterized by FTIR, and, since it did not introduce perturbing probes, the structure of lipids and proteins were simultaneously studied in intact biological membranes. Information on the secondary structure of peptides can be derived from the analysis of the strong amide I band. Orientation of secondary structural elements within a lipid bilayer matrix can be determined by means of polarized attenuated total reflectance-FTIR spectroscopy (ATR-FTIR). This new
technique is widely used in analysis of the structures of proteins from biological materials that cannot be studied by x-ray crystallography and NMR. The principle of ATR-FTIR is based on the measurement of change of the orientation of dipole from the change of electric fields applied (Vigano et al. 2000). FTIR spectroscopy is a sensitive tool to identify α-helical and β-sheet structures and changes in the structure on interaction with charged lipids. Polarized IR spectroscopy reveals that the antiparallel β-sheet structures oriented parallel to the membrane surface.

Besides, with the synthesis of peptides becoming increasingly popular nowadays, FTIR spectroscopy has been used to analyze the structure of synthetic peptides corresponding to functionally/structurally important regions of large proteins. The change in secondary structures caused by aggregation can also be detected by FTIR (Haris and Chapman 1995).

**Raman Spectroscopy**

Raman spectroscopy is a related vibrational spectroscopic method. It has a different mechanism and therefore can provide complementary information to infrared absorption for the peptide protein conformational structure determination and some multicomponent qualitative and/or quantitative analysis (Alix et al. 1985).

Raman spectroscopy measures the wavelength and intensity of inelastically scattered monochromatic radiation light from molecules. The Raman scattered light occurs at wavelengths that are shifted from the incident light by the energies of molecular vibrations. The spectra result from change in polarizability during the molecular vibrations. The most common light source in Raman spectroscopy is an Ar-ion laser. Raman spectroscopy requires tunable radiation and sources are Ar-ion-laser-pumped dye lasers, or high-repetition-rate excimer-laser-pumped pulsed dye lasers. Raman scattering is a weak process, therefore, the spectrometer must provide a high rejection of scattered laser light in order to get the spectra. New methods such as very narrow rejection filters and Fourier transform techniques are becoming more widespread.

In the literature Raman spectroscopy has been used to characterize protein secondary structure using reference intensity profile method (Alix et al. 1985). A set of 17 proteins was studied with this method and results of characterization of secondary structures were compared to the results obtained by x-ray crystallography methods. Deconvolution of the Raman Amide I band, 1630–1700 cm⁻¹, was made to quantitatively analyze structures of proteins. This method was used on a reference set of 17 proteins, and the results show fairly good correlations between the two methods (Alix et al. 1985).

**Atomic Absorption (AA) Spectrophotometry**

Atomic absorption spectrophotometry was developed in the 1950s by Dr. Alan Walsh.¹ The instrumentation of this method is shown in Figure 6.1. In general, chemical compounds are converted into their atomic constituents, and then the light absorption at a wavelength characteristic of a particular atomic species is determined.

¹ http://www.chem.uwa.edu.au/enrolled_students/MAST_sect2/sect2.5.2.html
and related to the concentration of the element in the sample from which it originated. The method to convert the analyte into its atomic form and a sensitive method for detecting light absorption over a very narrow wavelength range require sophisticated instrument design and experimental procedures. It exploits the narrowness of atomic absorption lines to avoid the necessity to separate a complex mixture prior to the analysis of its components.

It is a method of elemental analysis for various practical reasons and it is essentially suitable for analysis only of metals. A large number of elements can be analyzed for at trace levels. Therefore, its biotechnologic applications mainly involve in measurement of inorganic elements such as alkali, trace, and heavy metals in biological investigations. It is also used in industry to monitor contaminating inorganic elements in bioreactors fermentation process and preparation of culture media.

**Mass Spectrometry (MS)**

The scope of the use of mass spectrometry in the protein analysis has grown enormously in the past few decades. MS has become an important analytical tool in biological and biochemical research. Its speed, accuracy and sensitivity are unmatched by conventional analytical techniques. The variety of ionization methods permits the analysis of peptide or protein molecules from below 500 Da to as big as 300 Da (Biemann 1990; Lahm and Langen 2000). Basically, a mass spectrometer is an instrument that produces ions and separates them in the gas phase according to their mass-to-charge ratio \( m/z \). The basic principle of operation is to introduce sample to volatilization and ionization source, and then the molecular fragments from the ionization of the sample are detected by various kinds of detector and the data are analyzed with computer software.

There are many different ionization techniques available to produce charged molecules in the gas phase, ranging from simple electron (impact) ionization (EI) and chemical ionization (CI) to a variety of desorption ionization techniques with acronyms such as fast atom bombardment (FAB), plasma desorption (PD), electrospray (ES), and matrix-assisted laser desorption ionization (MALDI) (Mano and Goto 2003).

In the early 1980s, ionization techniques such as FAB, PD, and thermospray (TSP) made it possible to use MS in analysis of high-mass macromolecules since the production of gas phase ions from charged and polar compounds can be done without prior chemical derivatization. FAB is a soft ionization technique that performs well...
for polar and thermally labile compounds. FABMS can analyze polypeptides up to 3–3.5 kDa and can extend the capability to 10–15 kDa with improvement of the instrument (Beranova-Giorgianni and Desiderio 1997). FABMS and liquid secondary ion mass spectrometry (LSIMS) techniques are useful in characterization of disulfide bonds and the glycosylation sites in proteins, which are very difficult to do by other techniques (Monegier et al. 1991).

Over the last decade or so, new ionization techniques such as ES and MALDI have been introduced and have increased still further the use of mass spectrometry in biology. Identification of proteins and characterization of their primary structure is a rapidly growing field in the postgenomic era. ES ionization was the first method to extend the useful mass range of instruments to well over 50 kDa (Mano and Goto 2003). The sample is usually dissolved in a mixture of water and organic solvent, commonly methanol, isopropanol, or acetonitrile. It can be directly infused, or injected into a continuous flow of this mixture, or be contained in the effluent of an HPLC column or CE capillary. First introduced in late 1980s, MALDI is a soft ionization technique that allows the analysis of intact molecules of high masses. It allows determination of the molecular mass of macromolecules such as peptides and proteins more than 300 kDa in size.

Matrix-assisted laser desorption ionization-time of flight (MALDI-TOFF) mass spectrometry, which was first invented about a decade ago, has become, in recent years, a tool of choice for large molecule analyses (Leushner 2001). In the past, the mass of larger biomolecules (>1–2 kDa) could only be approximately determined using gel electrophoresis or gel permeation chromatography. The MALDI soft ionization technique coupled with a TOFF detector measuring flight time of the charged molecules in applied electric field make it possible to determine the exact mass of large protein molecules (Kevin 2000). This platform is ideal for analysis of protein and nucleic acid sequence, structure and purity. MALDI-TOFF is the method of choice for quality assurance in oligonucleotides and peptide synthesis (Leushner 2001). MALDI-TOFF MS is a very fast and sensitive technique, implemented on small, relatively inexpensive instruments that do not require extensive expertise in mass spectrometry. Such instruments are ideally suited for biological scientists who need molecular mass information more quickly and more accurately than can be obtained by gel electrophoresis.

MS is the most important tool to study post-translational modifications including partial proteolytic hydrolysis, glycosylation, acylation, phosphorylation, cross-linking through disulfide bridges, etc of the proteins (Jonsson 2001). These modifications usually result in the functional complexity of proteins.

Characterization of noncovalent bonding of the proteins can also be done using MS. For example MALDI MS has been used in measurement of the molecular mass of the noncovalently linked tetramer of glucose isomerase, a complex consisting of identical monomers of 43.1 kDa each. MALDI-TOFF peptide mass fingerprinting combined with electrospray tandem mass spectrometry can efficiently solve many complicated peptide protein analysis problems.

Affinity capture-release electrospray ionization mass spectrometry (ACESIMS) and isotope-coded affinity tags (ICAT) are two recently introduced techniques for the quantitation of protein activity and content with applications to clinical enzymology.
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and functional proteomics, respectively. Another common feature of the ACESIMS and ICAT approaches is that both use conjugates labeled with stable heavy isotopes as internal standards for quantitation (Turecek 2002).

Phosphorylation on serine, threonine, and tyrosine residues is an extremely important modulator of protein function. Phosphorylation can be analyzed by mass spectrometry with enrichment of compounds of interest using immobilized metal affinity chromatography and chemical tagging techniques, detection of phosphopeptides using mass mapping and precursor ion scans, localization of phosphorylation sites by peptide sequencing, and quantitation of phosphorylation by the introduction of mass tags (McLachlin and Chait 2001).

Recent developments in instrument design have led to lower limits of detection, while new ion activation techniques and improved understanding of gas-phase ion chemistry have enhanced the capabilities of tandem mass spectrometry for peptide and protein structure elucidation. Future developments must address the understanding of protein–protein interactions and the characterization of the dynamic proteome (Chalmers and Gaskell 2000).

New instrumentation for the analysis of the proteome has been developed including a MALDI hybrid quadrupole time of flight instrument which combines advantages of the mass finger printing and peptide sequencing methods for protein identification (Andersen and Mann 2000).

Electrospray in the mid 1980s revolutionized biological mass spectrometry, in particular in the field of protein and peptide sequence analysis. Electrospray is a concentration-dependent, rather than a mass-dependent process, and maximum sensitivity is achieved at low flow rates with high-concentration, low-volume samples (Griffiths 2000). Joint NMR, x-ray diffraction, electrophoresis, and chromatography techniques with mass spectrometry (MS) techniques would be a trend in the future.

NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY (NMR)

NMR analysis allows characterization of proteins to an atomic level. The most frequently used nuclei on protein NMR are $^1$H, $^2$H, $^{13}$C, $^{15}$N, and $^{17}$O with proton NMR (Jefson, 1988). The use of NMR methods for protein sequence and conformational studies was limited to the small proteins or peptides because high magnetic fields were required but not widely available to study larger molecules and it was very time consuming with the capability of instruments in the past.

As the recent development of the high-field superconducting magnet systems and improvement in computer hardware and software, the use of 2-dimensional or 2D-NMR techniques becomes feasible and has made it possible to analyze larger protein molecules (Nagayama 1981). Furthermore, the line broadening effect from large proteins and the nonisotropic motion of proteins in the lipid matrix will affect the resulting chromatogram, therefore, the identification of structures. Solid-state NMR spectroscopy can be used to analyze structures of proteins or peptides interact with lipid membranes (Bechinger 1999). Detailed structure analysis of these oligosaccharides conjugated to the proteins can be accomplished by high field NMR, providing anomeric configurations and linkage information.
Electron Spin Resonance

Electron spin resonance (ESR) spectroscopy has been used for more than 50 years to study a variety of paramagnetic species. ESR or electron paramagnetic resonance (EPR) spectroscopy measures the presence of free radicals in solution and also gains information about the environment surrounding the free radical species (Sealy 1985). The electron spin resonance spectrum of a free radical or coordination complex with one unpaired electron is the simplest of all forms of spectroscopy. The degeneracy of the electron spin states characterized by the quantum number, \( m_s = \pm 1/2 \), is lifted by the application of a magnetic field and transitions between the spin levels are induced by radiation of the appropriate frequency. The integrated intensity in the spectrum is proportional to the radical concentration from both unpaired and free electrons. An unpaired electron interacting with its environment also affects the details of ESR spectra. Several studies investigated the role of certain metal ions in enzyme mechanisms (Pellegrini and Mierke 1999; Hoffman, 1991). These studies are further enhanced by the technique known as electron nuclear double resonance (ENDOR) of metalloenzymes. An ENDOR experiment provides an NMR spectrum of those nuclei that interact with the electron spin of a paramagnetic center. ESR has its limitation in characterization of proteins that are of interest as biotechnology products. It can only analyze proteins that contain a paramagnetic metal ion or other source of unshared electrons. Therefore, ESR spectroscopy is useful to characterize enzymes that require a paramagnetic metal for its catalytic activity. The information about the presence of the metal and its oxidation state can be obtained through ESR.

Optical Rotary Dispersion and Circular Dichroism Spectroscopy

Circular dichroism (CD) is a spectroscopic analytical technique used for conformational analysis of peptides and proteins (Johson 1988). It uses the principles of chirality and absorption; specifically the different absorption profiles demonstrated by a system for left as opposed to right circularly polarized light. For a system to exhibit CD activity, it must contain a chiral (asymmetric) center that is linked in some way to the chromophore responsible for the absorption.

Rotation of the polarization plane (or the axes of the dichroic ellipse) by a small angle \( \alpha \) occurs when the phases for the two circular components become different, which requires a difference in the refractive index \( n \) (Pearlman and Nguyen 1991). This effect is called circular birefringence. The change of optical rotation with wavelength is called optical rotary dispersion (ORD).

Biologically active molecules often have both requirements. Disulfide groups, amide groups in the peptide backbone, and aromatic amino acids provide the UV absorption required to acquire a CD spectrum (Ettinger and Timasheff 1971). The units are measured in \( \text{cm}^2/\text{dmol} \). Two major benefits to using this technique are that it only requires a small amount of protein and it takes less than 30 minutes to process the sample. The application of circular dichroism with regard to the analysis of peptides and proteins focuses on determining secondary structure: \( \alpha \)-helices and \( \beta \)-sheets, and gives some information regarding changes in tertiary structure (Pearlman and Nguyen 1991; Ettinger and Timasheff 1971a,b). The far UV region (190–250 nm) usually corresponds to the secondary structure and changes in the near UV region are
considered to correspond to changes in the tertiary structure. α-Helices have a distinct positive shoulder ~175 nm, with a crossover ~170 nm and a negative peak below 170 nm. β-Sheets have a crossover at ~185 nm and a strong negative peak between 170 and 180 nm (Pearlman and Nguyen 1991; Ettinger and Timasheff 1971a).

Further information is available from the vacuum ultraviolet region (VUV, below 190 nm), although measurement in this region is difficult with most conventional lab CD equipment due to the low intensity of the light source and the high absorption of the sample, buffer, and solvent. Synchrotron radiation CD has been used to determine protein spectra in aqueous solutions to wavelengths as low as 160 nm; it is hoped that this will find a useful application in structural genomics. CD spectra are sensitive to changes in temperature, pH, solvent, and ionic strength among others. With optimized conditions, CD spectra can serve as unique identifiers for biological molecules (Kenney and Arakawa 1997).

**FLUORESCENCE SPECTROSCOPY**

**Chemiluminescence and Bioluminescence**

Colorimetric assays are commonly used in molecular biology and biotechnology laboratories for determining protein concentrations because the procedures and their instrumentation requirements are simple. Two forms of assays are used. The first involves reactions between the protein and a suitable chemical to yield a colored, fluorescent, or chemiluminescence product. Second, a colored dye is bound to the protein and the absorbance shift is observed. Disadvantages of both these methods include limited sensitivity at below 1 µg/mL, interferences from buffers, and unstable chromophores (Jain et al. 1992).

Luminescence spectroscopy involves three related optical methods: fluorescence, phosphorescence, and chemiluminescence. These methods utilize excited molecules of an analyte to give a species whose emission spectrum can provide information about the molecule. In fluorescence, atoms can be excited to a higher energy level by the absorption of photons of radiation. Some features of luminescence methods are increased sensitivity (in the order of three magnitudes smaller than absorption spectroscopy), larger linear range of concentration, and method selectivity (Parsons 1982).

Fluorescence determinations are important to analyze cysteine, guanidine, proteins, (LSD), steroids, a number of enzymes and coenzymes, and some vitamins, as well as several hundred more substances. A fluorometer can be used to verify conformational changes in multiparticle operator recognition by λ-repressor as explained in a journal article by Deb et al. (2000). Upon titration with single operators site, the tryptophan fluorescence quenches to different degrees, suggesting different conformations of the DNA-protein complexes.

Phosphorimetric methods have been used to determine such substances as nucleic acids, amino acids, and enzymes. However, this is not a widely used method since it cannot be run at room temperature. Measurements are usually performed with liquid nitrogen to prevent degradation due to collision deactivation. Fluorometric methods are used to determine both inorganic and organic species. Instruments used for measuring fluorescence and phosphorescence are fluorometers and spectrophotometers, respectively. These instruments are similar to ultraviolet and visible spectrometers,
except for some differences in both the source and detector. The source must be more intense than the deuterium or tungsten lamps and therefore usually use either mercury or xenon arc lamps or lasers to excite the source. The detector signal usually requires amplification due to the low intensity of the fluorescent signal.

Chemiluminescence chemical reactions are found in several biological systems in which light is emitted from the excited species as it returns to the ground state. Often, treatment of samples with fluorescence labeling agent reacts with primary and secondary amines to give a fluorescent compound. This is especially important for detecting amino acids in protein hydrolyzates. Fluorescence detectors may also be integrated into a high performance liquid chromatographic (HPLC) system.

**X-Ray Crystallography**

X-ray crystallographic analysis is a spectroscopic experimental method utilized for the three-dimensional analysis of proteins. This technique involves an x-ray beam bombarding a crystalline lattice in a given orientation and the measurement of the resulting diffraction pattern and intensity which is attributable to the atomic structure of the lattice (Palmer and Niwa 2003). It is important to note that in order to resolve the structure of a macromolecule using x-rays, it must be crystallized and the crystals must be singular. Molecules with highly hydrophobic portions such as the transmembrane portions of proteins are very difficult to crystallize, although crystals may be obtained in some cases using detergents.

An x-ray analysis will measure the diffraction pattern (positions and intensities) and the phases of the waves that formed each spot in the pattern. These parameters combined result in a three-dimensional image of the electron clouds of the molecule, known as an electron density map. A molecular model of the sequence of amino acids, which must be previously identified, is fitted to the electron density map and a series of refinements are performed. A complication arises if disorder or thermal motion exist in areas of the protein crystal; this makes it difficult or impossible to discern the three-dimensional structure (Perczel et al. 2003).

Time-resolved x-ray crystallography (TC) is a more recent advanced application of x-ray crystallography. It uses an intense synchrotron x-ray source and data collection methods to reduce crystallographic exposure times. This allows multiple exposures to be taken over time at near-physiological, crystalline conditions to determine the structures of intermediates. A typical problem with this method is that the existence of the intermediates is brief, resulting in difficulty in interpreting the resulting electron density maps.

**Dynamic Light Scattering**

Dynamic light scattering (DLS), also called photon correlation spectroscopy (PCS) or quasi-elastic light scattering (QELS), can be used for comparing the stability of different formulations, including monitoring of changes at increasing temperatures (Martindale et al. 1982). Upon degradation many proteins start to form aggregates. The appearance of these aggregates may take several months. DLS is used to detect the precursory aggregates too small to be detected by the naked eye. Researchers
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must know the rate at which proteins start to degrade and experiment with various solutions in order to find the formulation that has the longest shelf life. However, if the particulates can be detected and identified early then formation may be prevented or minimized (Tsai et al. 1993).

DLS uses the scattered light from the protein to measure the rate of diffusion of the protein particles. The instrument, DynaPro MS/X measures the time dependence of the light scattered from a small region of solution over a short time range. The instrument is based on Brownian motion. Brownian motion is the observed movement of small particles that are randomly bombarded by the molecules of the surrounding medium. The changes in the intensity of the light are related to the rate of diffusion of the molecules in and out of that small region of solution. The information is plotted in a graph of the size of the particle radius (nm) vs. intensity (%) (Kadima et al. 1993). One of the largest shortcomings of this technique is that it is difficult to quantitate the amount of aggregates. Therefore, it is used primarily for the comparison of different formulations.

OTHER METHODS

Differential Scanning Calorimetry (DSC)

Differential scanning calorimetry is primarily used to determine changes in proteins as a function of temperature. The instrument used is a thermal analysis system, for example a Mettler DSC model 821e. The instrument coupled with a computer can quickly provide a thermal analysis of the protein solution and a control solution (no protein). The instrument contains two pans with separate heaters underneath each pan, one for the protein solution and one for the control solution that contains no protein. Each pan is heated at a predetermined equal rate. The pan with the protein will take more heat to keep the temperature of this pan increasing at the same rate of the control pan. The DSC instrument determines the amount of heat (energy) the sample pan heater has to put out to keep the rates equal. The computer graphs the temperature as a function of the difference in heat output from both pans. Through a series of equations, the heat capacity ($C_p$) can be determined (Freire 1995).

As the temperature of a protein solution is increased the protein will begin to denature. Various protein formulations can be compared to see which has the greatest resistance to temperature. The formulation may vary by testing different agents to stabilize the protein and inhibit denaturation. Creveld et al. (2001) used DSC to determine the stability of *Fusarium solani pisi* cutinase (an enzyme used in laundry detergent) when used in conjunction with surfactants. They concluded that sodium taurodeoxycholate (TDOC) stabilized the unfolding state of the enzyme and in return lowered the unfolding temperature and made the unfolding reversible.

CHROMATOGRAPHIC METHODS

Chromatography is a broad range of techniques that study the separation of molecules based on differences in their structure and/or composition. In general, chromatography involves a sample being dissolved in a mobile phase (which may be a gas, a liquid,
or a supercritical fluid). The mobile phase is then forced through an immobile, immiscible stationary phase. The molecules in the sample will have different interactions with the stationary support leading to separation of similar molecules. Test molecules, which display tighter interactions with the support will tend to move more slowly through the support than those molecules with weaker interactions. In this way, different types of molecules can be separated from each other as they move over the support material.

Based on the mechanism of the separation, several different types of chromatography (partition, adsorption, ion exchange [IEC], and size exclusion [SEC]) are available to biotechnologists for verifying protein stability. These techniques are summarized below.

**Adsorption Chromatography**

Adsorption chromatography relies on the different affinity of components of a mixture for a liquid moving phase and a solid stationary phase. The separation mechanism depends upon differences in polarity between the different feed components. The more polar a molecule, the more strongly it will be adsorbed by a polar stationary phase (Varki et al. 1999). Similarly, the more nonpolar a molecule, the more strongly it will be adsorbed by nonpolar stationary phase. It is often employed for relatively nonpolar, hydrophobic materials so that the solvent tends to be nonpolar while the stationary phase is polar. Proteins have a high affinity to polar chromatographic media and the recovery of the sample is usually difficult. Therefore, this method is not commonly used to purify and characterize proteins.

Recently, a new developed adsorption chromatography named expanded bed adsorption chromatography has been proved a very powerful technique for capture of proteins directly from unclarified crude sample. Ollivier and Wallet (19XX) described the successful high-yield purification of a recombinant therapeutic protein in compliance with current Good Manufacturing Practice (cGMP) using expanded bed adsorption chromatography.

**Gas-Liquid Chromatography (GLC)**

Gas-liquid chromatography is a chromatographic technique that can be used to separate volatile organic compounds (48). As shown in Figure 6.2, a gas chromatograph consists of a flowing mobile phase, an injection port, a separation column containing the stationary phase, and a detector. The organic compounds are separated due to differences in their partitioning behavior between the mobile gas phase and the stationary phase in the column. Gas-liquid chromatography makes use of a pressurized gas cylinder and a carrier gas, such as helium, to carry the solute through the column. In this type of column, an inert porous solid is coated with a viscous liquid, which acts as the stationary phase. Diatomaceous earth is the most common solid used. Solutes in the feed stream dissolve into the liquid phase and eventually vaporize. The separation is thus based on relative volatilities. The most common detectors used in this type of chromatography are thermal conductivity and flame ionization detectors. In the latter, the compounds are ionized in a flame, the ions are collected on electrodes, and the quantity of ions collected is determined by the
perturbation of the voltage in the electrode circuit. The chromatogram consists of peaks relative to time after injection of the sample onto the column. Peak areas (in arbitrary units) are usually determined with an integrator. Personal computer programs are available for online identification and quantitation of compounds.

Gas-liquid chromatography used for the determination of C-terminal amino acids and C-terminal amino acid sequences in nanomolar amounts of proteins was described in 1976 by Davy and Morris. Based on carboxypeptidase A digestion of the protein, the partially digested protein was removed and the amino acids released after known time intervals were analyzed by quantitative gas-liquid chromatography. Sequences deduced from the kinetics of release of specific amino acids are compared with the known C-terminal sequences of well-characterized proteins. Thus the amino acid sequences were determined.

**PARTITION CHROMATOGRAPHY**

The basis of this chromatography is the partition of solutes between two immiscible liquid phases, one stationary and the other mobile (Figure 6.3). In general, if two phases are in contact with one another and one phase contains a solute, the solute will distribute itself between the two phases according to its physical and chemical properties, which is called the partition coefficient (the ratio of the concentrations of the solute in the two phases). With mobile liquid phases, there is a tendency for the stationary liquid phase to be stripped or dissolved (Muller 1990). Therefore, the stationary liquid phase has to be chemically bonded to the solid bonding support. In partition chromatography, the stationary liquid phase is coated onto a solid support such as silica gel, cellulose powder, or hydrated silica. Assuming that there is no adsorption by the solid support, the feed components move through the system at
rates determined by their relative solubility in the stationary and mobile phases. In general, it is not necessary for the stationary and mobile phases to be totally immiscible, but a low degree of mutual solubility is desirable. Hydrophilic stationary phase liquids are generally used in conjunction with hydrophobic mobile phases (referred to as “normal-phase chromatography”) (Abbott 1980), or vice versa (referred to as a “reverse-phase chromatography”) (Smith 1967). Suitable hydrophilic mobile phases include water, aqueous buffers, and alcohols. Hydrophobic mobile phases include hydrocarbons in combination with ethers, esters, and chlorinated solvents. Partition chromatography is used primarily for proteins of small molecular weight.

Partition chromatography is of great importance for ascertaining the sequence of amino-acid residues in the peptide chains of proteins. If a peptide chain is partially degraded to dipeptide and tripeptide fragments, and so forth it should be possible, by identifying these, to recognize the original compound from which they are derived. Sanger and colleagues (1959) elucidated by partition-chromatographic methods what may be the entire peptide sequences in the structure of ox insulin, the minimum molecule of which embodies 51 amino-acid residues.

**ION EXCHANGE CHROMATOGRAPHY**

Ion exchange chromatography is applicable to charged solutes which can be separated on the basis of their strength of binding of oppositely charged groups presented on the stationary phase (Figure 6.4). It is a special type of adsorption chromatography in which the adsorption is very strong (Gold 1997). Charged molecules adsorb to ion exchangers readily and can be eluted by changing the ionic environment.

Ion exchange chromatography is commonly used in the purification of biological materials. There are two types of exchange: cation exchange in which the stationary phase carries a negative charge, and anion exchange in which the stationary phase carries a positive charge. Charged molecules in the liquid phase pass through the column until a binding site in the stationary phase appears. The molecule will not elute from the column until a solution of varying pH or ionic strength is passed through it. The degree of affinity between the stationary phase and feed ions dictates the rate of migration and hence degree of separation between the different solute species. The most widely used type of stationary phase is a synthetic copolymer of styrene and divinyl benzene (DVB), produced as very small beads in the micrometer range. Careful
control over the amount of DVB added dictates the degree of cross-linking and hence the porosity of the resinous structure (Yang and Regnier 1991). Resins with a low degree of cross-linking have large pores that allow the diffusion of large ions into the resin beads and facilitate rapid ion exchange. Highly cross-linked resins have pores of sizes similar to those of small ions. The choice of a particular resin depends on a given application. Cation or anion exchange properties can be introduced by chemical modification of the resin.

Ion exchange chromatography is probably the most widely used large-scale chromatographic process. Separation of proteins by this method is highly selective since the resins are fairly inexpensive and high capacities can be used. It has widespread uses in industrial processes. Ion-exchange chromatography is an extremely common protein purification technique. The majority of proteins are separated using anion, rather than cation IEC, probably because most proteins have an acidic pl. De et al. (2002) used anion IEC to separate a lethal neurotoxic protein from cobra venom. In their study, proteins were loaded onto the CM-Sephadex column at around pH 7 and eluted by increasing the amount of salt. Changing the pH could elute the targeted proteins. Thus the toxin was isolated and purified.

**SIZE-EXCLUSION CHROMATOGRAPHY (SEC)**

Size-exclusion chromatography (SEC) also known as gel permeation chromatography, uses porous particles to separate molecules of different sizes or molecular weight. The use of SEC and IEC is well suited for use with biologically active proteins since each protein has its own unique structure and the techniques may be performed in physiological conditions (Liu et al. 2002). The retention of components is based on the size in solution. The largest molecules are excluded from the stationary phase pores and elute earlier in the chromatogram. Molecules that are smaller than the pore size can enter the particles and therefore have a longer path and longer time than larger molecules that cannot enter the particles (Barth et al. 1994) (Figure 6.5). The stationary phase of SEC consists of a porous cross-linked polymeric gel. The pores of the gel vary in size and shape such that large molecules tend to be excluded by the smaller pores and move preferentially with the mobile
The smaller molecules are able to diffuse into and out of the smaller pores and will thus be slowed down by the system. The components of a mixture therefore elute in order of decreasing size or molecular weight. The stationary phase gels can either be hydrophilic for separations in aqueous or polar solvents, or hydrophobic for use with nonpolar or weakly polar solvents. Sephadex® (Sigma Aldrich Co, St. Louis, MO) a cross-linked polysaccharide material available in bead form, is widely used with polar/hydrophilic mobile phases. The degree of cross-linking can be varied to produce beads with a range of pore sizes to fractionate samples over different molecular weight ranges. Hydrophobic gels are made by cross-linking polystyrene with divinyl benzene (DVB) and are therefore similar to ion exchange resins but without the ionic groups.

The principal feature of SEC is its gentle noninteraction with the sample, enabling high retention of biomolecular enzymatic activity while separating multimers that are not easily distinguished by other chromatographic methods. SEC is used extensively in the biochemical industry to remove small molecules and inorganic salts from valuable higher molecular weight products such as peptides, proteins and, enzymes. SEC is also used to determine the molecular weight and molecular weight distribution of a number of polymers such as polycarbonate, polyurethane and organopolysiloxanes.

Size-exclusion chromatography can be used to analyze protein–protein interactions. Bloustine et al. (2003) presented a method to determine second virial coefficients (B2) of protein solutions from retention time measurements in size-exclusion chromatography.
chromatography. B2 was determined by analyzing the concentration dependence of the chromatographic partition coefficient. This method was able to track the evolution of B2 from positive to negative values in lysozyme and bovine serum albumin solutions. The size-exclusion chromatography results agree quantitatively with data obtained by light scattering.

**Affinity Chromatography**

Affinity chromatography involves the use of packing that has been chemically modified by attaching a compound with a specific affinity for the desired molecules, primarily biological compounds (Wilchek and Chaiken 2000). The packing material used, called the affinity matrix, must be inert and easily modified. Agarose is the most common substance used, despite its cost. The ligands that are inserted into the matrix can be genetically engineered to possess a specific affinity. Successful separation by affinity chromatography requires that a biospecific ligand is available and that it can be covalently attached to a matrix. It is important that the biospecific ligand (antibody, enzyme, or receptor protein) retains its specific binding affinity for the substance of interest (antigen, substrate, or hormone) (Kent 1999) (Figure 6.6).

Affinity chromatography is widely used as a means of separation and purification with specific properties. It represents one of the most effective methods for the purification of proteins as well as many other molecules. For example, Loog et al. (2000) used affinity ligands, which consist of ATP- resembling part coupled with specificity determining peptide fragment, to purify protein kinases. Affinity sorbents, based on two closely similar ligands AdoC-Aoc-Arg4-Lys and AdoC-Aoc-Arg4-NH(CH2)6NH2, were synthesized and tested for purification of recombinant protein kinase A catalytic subunit directly from crude cell extract. Elution of the enzyme

![Affinity Chromatography Diagram](image-url)

**FIGURE 6.6** Affinity chromatography uses antigen–antibody binding to purify antigens or antibodies. To purify a specific antigen from a complex mixture of molecules, a monoclonal antibody is attached to an insoluble matrix, such as chromatography beads, and the mixture of molecules is passed over the matrix. The specific antibody binds the antigen of interest; other molecules are washed away. Altering the pH, which can usually disrupt antibody–antigen bonds, then elutes specific antigen. Antibodies can be purified in the same way on beads coupled to antigen.
with MgATP as well as L-arginine yielded homogeneous protein kinase A preparation in a single purification step. The affinity ligand was highly selective. Protein kinase with acidic specificity determinant (CK2) as well as other proteins possessing nucleotide binding site (L-type pyruvate kinase) or sites for wide variety of different ligands (bovine serum albumin) did not bind to the column.

Purification of antibodies from animal sera is another common use of affinity chromatography. Sun et al. (2003) produced a peptide affinity column by employing intein-mediated protein ligation (IPL) in conjunction with chitin affinity chromatography. Peptide epitopes possessing an N-terminal cysteine were ligated to the chitin bound CBD tag. The resulting peptide columns permit the highly specific and efficient affinity purification of antibodies from animal sera.

**HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY**

The application of HPLC to biomolecules began in the mid-1970s and is preferred because of its speed and resolution. Since the native protein (bioactive) molecule has a well-defined tertiary or quaternary structure, during method validation the HPLC conditions are dependent on the protein conformation (Figure 6.7). Varying HPLC conditions such as the HPLC method, mobile phase composition, the nature of the packing material, flow rate, and separation temperature are all critical to the analysis of the proteins stability. Changes in conformation that occur during or after separation usually result in an unsatisfactory separation. The ionic charge of the peptide molecule has an important role in the separation and can be controlled by varying mobile phase pH. pKa values of side chains and terminal amino acids may be altered in the native protein. The perseverance of the native conformation (and bioactivity) is the primary
consideration in method development. The following are examples of how bioactivity can be preserved: restricted pH range of the mobile phase, limited concentration of organic solvent, low temperatures, ionic strengths, presence or absence of metal ions, substrates, or products, detergents, and lastly, range of the bulk protein. Reasons for poor recovery can be attributed to partial denaturation and irreversible attachment to particle matrix. The detectors utilized by chromatographic systems are ultraviolet absorption, infrared absorption, fluorometry and LC coupled with mass spectrometry as well as others. Fluorescence detection can be more sensitive and selective but is used less universally (Fukushima et al. 2003).

HPLC was used by Lu and Chang (2001) to identify conformational isomers of mPrP(23-231). The data produced from the study indicated that the reduced form of mouse prion protein was able to exist as at least four diverse isoforms and were able to be separated by HPLC. More importantly, this technique facilitated the isolation of the isomers and confirmation of the protein molecule.

ELECTROPHORESIS

Electrophoresis is a commonly used technique in the analysis of peptides and proteins. This technique refers to the migration of charged particles when dissolved or suspended in an electrolyte through which an electric current is passed. Cations migrate toward the negatively charged electrode (cathode), while anions are attracted toward the positively charged electrode (anode). Neutral particles are not attracted toward either electrode. The migration observed for proteins is dependent on its size, shape, electrical charge, and molecular weight, as well as characteristics and operating parameters of the system. Parameters of the system include the pH, ionic strength, viscosity and temperature of the electrolyte, density or cross-linking of any stabilizing matrix such as a gel, and the voltage gradient employed. The rate of migration is directly related to the magnitude of the net charge on the particle and is inversely related to the size of the particle, which in turn is directly related to its molecular weight (Goldenberg and Creighton 1984; West et al. 1984).

Common types of electrophoresis include gel electrophoresis, isoelectric focusing and capillary methods (Strege and Lagu 1993). In gel electrophoresis, analytical processes employ a gel such as agar, starch, or polyacrylamide as a stabilizing medium. The method is particularly advantageous for protein separations because of its high resolving power. The separation obtained depends upon the electrical charge to size ratio coupled with a molecular sieving effect dependent primarily on the molecular weight.

One of the most widely used forms of gel electrophoresis is known as sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Polyacrylamide gel has several advantages that account for its extensive use. It has minimal adsorptive properties and produces a negligible electroosmotic effect (HjelmeLand and ChrAmbach 1981). In identity tests, for the determination of molecular weight, SDS-PAGE has been shown to be an appropriate, fast, and easy method that is often used in quality control laboratories. The use of SDS-PAGE followed by a densitometric analysis, such as MS, is a helpful technique for the determination of peptide or
protein mass (Westbrook et al. 2001). Blots (Southern, Northern, and Western) are an extension of SDS-PAGE and are especially useful in biotechnology. Among them Western blots are used for proteins rather than nucleic acids. In this method, following electrophoresis the resolved proteins are transferred onto a nitrocellulose or polyvinylidene difluoride membrane and reacted with an antibody. The complex then can be visualized using an enzymatically labeled or radioactively labeled antibody as explained in the immunassay section.

As the focus of the pharmaceutical industry turns toward the functional use of proteins, the use of two-dimensional (2D) gel electrophoresis followed by mass spectrometry has become the norm for the quantitative measurement of gene expression at the protein level (Davy and Morris 1976). Recent developments in this field aim to enhance the staining technology and the recovery of peptides from gel digests for mass spectrometric identification. Advances in this area include the use of a novel, ruthenium-based fluorescent dye, SYPRO Ruby Protein Gel Stain, for the detection of proteins in SDS-PAGE. Since the linear dynamic range of SYPRO Ruby Protein is orders of magnitude greater than conventional stains, quantitative differences, especially in low-abundance proteins are easier to detect (Nishihara and Champion 2002). This improved capability is of significant importance in differential protein expression studies where a small increase or decrease in the abundance of a protein isoform might allow the early detection of disease.

Isoelectric focusing separates proteins according to their respective isoelectric points (pI, the pH at which proteins have no charge). It can also be used to test the stability of a protein since the deamidation leads to the production of a new carboxylic acid group, resulting in a shift of pI toward the acidic side (Welinder 1971; Kosecki 1988).

Another electrophoretic method that has become quite popular recently is capillary electrophoresis (CE). The use of capillaries as a migration channel in electrophoresis has enabled analysts to perform electrophoretic separations on an instrumental level comparable to that of high-performance liquid chromatography (Strege and Lagu 1993; Rabel and Stobaugh 1993). CE-based analytical procedures have advantages such as high separation efficiency, short run-time, instrumentation simplicity, minimum operation costs, and compatibility with small sample volumes. And as with 2D gel electrophoresis, the combination of a CE separation system with densitometric analysis has proved to be a useful combination. Researchers observed that the coupling of CE to matrix-assisted laser desorption/ionization TOF (time of flight flow) mass spectrometry (MALDI-TOF) greatly increases the total number of identifiable peptides when compared to MALDI-TOF techniques alone (Rubakhin et al. 1993). Even in the dairy industry, where the composition and integrity of proteins or peptide-derived proteins is important in determining a product’s value, CE proves to a central analytic technique as it provides rapid, high resolution separation and quantification of proteins and peptides without a need for prior separation (Righetti 2001).

During typical CE operation with an uncoated capillary filled with an operating buffer, silanol groups on the inner wall of the glass capillary release hydrogen ions to the buffer and the wall surface becomes negatively charged, even at a fairly low pH. Solutes having partial positive charges in the medium are attracted to the
negatively charged wall, forming an electrical double layer. Applying voltage across
the length of the capillary causes the solution portion of the electrical double layer
to move toward the cathode end of the capillary, drawing the bulk solution. This
movement is known as the electro-osmotic flow (EOF). The degree of ionization of
the inner-wall capillary silanol groups depends mainly on the pH of the operating
buffer and on the modifiers that may have been added to the electrolyte.

Currently, there are five major modes of operation of CE: capillary zone elec-
trophoresis (CZE), also referred to as free solution or free flow capillary electro-
phoresis; micellar electrokinetic chromatography (MEKC); capillary gel electro-
phoresis (CGE); capillary isoelectric focusing (CIEF); and capillary isotachophoresis
(CITP). Of these, the most commonly utilized capillary techniques are CZE and
MEKC (Rabel and Stobaugh 1993; Issaq 1999; Smyth and McClean 1998).

In CZE, separations are controlled by differences in the relative electrophoretic
mobilities of the individual components in the sample or test solution. The mobility
differences are functions of analyte charge and size under specific method conditions.
They are optimized by appropriate control of the composition of the buffer, its pH,
and its ionic strength.

In MEKC, the supporting electrolyte medium contains a surfactant at a concen-
tration above its critical micelle concentration (CMC). The surfactant self-aggregates
in the aqueous medium and forms micelles whose hydrophilic head groups and
hydrophobic tail groups form a nonpolar core into which the solutes can partition.
The micelles are anionic on their surface, and they migrate in the opposite direction
to the electroosmotic flow under the applied current. The differential partitioning of
neutral molecules between the buffered aqueous mobile phase and the micellar
pseudostationary phase is the sole basis for separation as the buffer and micelles
form a two-phase system, and the analyte partitions between them (Smyth and
McClean 1998).

In CIEF, proteins are separated on the basis of their relative differences in
isoelectric points. This is accomplished by achieving steady-state sample zones
within a buffer pH gradient, where the pH is low at the anode and high at the cathode.
With the application of an electric field, the proteins then migrate according to their
overall charge. This technique has proven to be useful in the characterization and
quality control of pharmaceutical peptides for separating and estimating the classes
of differently charged isoforms, as well as the detection of post-translational mod-
fications like glycosylation or phosphorylation (Shimura 2002).

CITP employs two buffers in which the analyte zone is enclosed between. Either
anions or cations can be analyzed in sharply separated zones. In addition, the analyte
concentrations are the same in each zone; thus, the length of each zone is proportional
to the amount of the particular analyte. And finally, CGE, which is analogous to gel
filtration, uses gel-filled capillaries to separate molecules on the basis of relative
differences in their respective molecular weight or molecular size. It was first used
for the separation of proteins, peptides, and oligomers. Advantages of the gel include
decreasing the electro-osmotic flow and also reducing the adsorption of protein onto
the inner wall of the capillary (von Brocke et al. 2001).

Capillary electrophoresis has become very successful in the bioanalysis of pro-
teins but researchers are often looking for ways to improve the various techniques,
as seen with the advances being researched in SDS-PAGE staining techniques. One complaint about CE is that, due to its low loading capacity, there is an intrinsically poor concentration sensitivity. Several solutions to increase sample loadability without resolution reduction, such as on-capillary preconcentration, have been made. The need for high-throughput screening systems (HTS) is becoming more significant and advances in CE such as the development of high-efficiency separation carriers for CGE methods. As the human genomic project moves into the postgenomic sequencing era, and the need for the accumulation of information on genes and proteins will be ever more increasing, advances such as the development of HTS systems for protein analysis could possibly one day allow for the bedside analysis of disease.

**BIOASSAYS**

**IMMUNOPRECIPITATION**

Immunoprecipitation (IP) is one of the most widely used immunochemical techniques. It involves the interaction between an antigen and its specific antibody. Antigen–antibody interactions may produce a network of many antigen molecules cross-linked by antibody molecules, which result in insolubilization and precipitation of the complex (Williams 2000).

Immunoprecipitation followed by SDS-PAGE and immunoblotting, is routinely used in a variety of applications: to determine the molecular weights of protein antigens, to study protein–protein interactions, to determine specific enzymatic activity, to monitor protein post-translational modifications and to determine the presence and quantity of proteins. The IP technique also enables the detection of rare proteins which otherwise would be difficult to detect since they can be concentrated up to 10,000-fold by immunoprecipitation (MacMillan-Crow and Thompson 1999). As usually practiced, this technique provides a rapid and simple means to analysis a protein of most interest. However the name of the procedure is a misnomer since removal of the protein antigen from solution does not depend upon the formation of an insoluble antibody–antigen complex. Rather, antibody–antigen complexes are removed from solution by addition of an insoluble form of an antibody binding protein such as protein A, protein G, or second antibody (Figure 6.8). Typically, the antigen is made radioactive before the immunoprecipitation procedure. Having a radioactive antigen is not required but interpretation of data is simplified since the antigen, and not the antibody, is radiolabeled.

The success of immunoprecipitation depends on the affinity of the antibody for its antigen as well as for protein G or protein A. In general, while polyclonal antibodies are best, purified monoclonal antibodies (MAb), ascites fluid, or hybridoma supernatant can also be used.

Immunoprecipitation can be used to study protein–DNA interactions (Kuo and Allis 1999). For instance, the basic chromatin immunoprecipitation technique is remarkably versatile and has now been used in a wide range of cell types, including budding yeast, fly, and human cells. This technique has been successfully employed to map the boundaries of specifically modified (e.g., acetylated) histones along target
genes, to define the cell cycle-regulated assembly of origin-dependent replication and centromere-specific complexes with remarkable precision, and to map the \textit{in vivo} position of reasonably rare transcription factors on cognate DNA sites.

**Complement Assay**

Complement is one of the triggered enzyme systems of serum proteins, its action usually initiated by the combination of antigen–antibody complex. The central reaction of the complement system can be regarded as the cleavage of C3 to C3a and C3b (Andersen and Mann 2000). C3 is the bulk component of the system and all the reactions of the system can be conveniently related to the cleavage, inactivation and subsequent part in the causation of the lytic lesion and other biological phenomena. The classical pathway of complement is activated by the binding of the C1q subunit of the C1 macromolecule to IgG or IgM that is aggregated with the antigen. This results in a cascade of proteins (Figure 6.9). Complement activation is customarily determined by measuring fixation of complement or complement-mediated haemolysis. Complement fixation is a test measured by the capacity of complement, after incubation with antigen–antibody complexes to hemolyze sensitized sheep red blood cells. The procedure involves some, if not all, of the components that comprise the complement system.

Complement fixation assays can be used to look for the presence of specific antibody or antigen in a patient’s serum. The test uses sheep red blood cells (SRBC), anti-SRBC antibody and complement, along with specific antigen (if looking for antibody in serum) or specific antibody (if looking for antigen in serum).
Pharmaceutical Biotechnology

If an antibody (or antigen) is present in the patient’s serum, then the complement is completely utilized and SRBC lysis is minimal. However, if the antibody (or antigen) is not present in the patient’s serum, then the complement binds anti-SRBC antibody and lysis of the SRBCs ensues. Color changes in the solution induced by RBC lysis can be measured spectrophotometrically (Figure 6.10).

**FIGURE 6.9** The classical pathway of complement activation is initiated by binding of C1q to antibody on a surface such as a bacterial surface. Multiple molecules of IgG bound on the surface of a pathogen allow the binding of a single molecule of C1q to two or more Fc pieces. The binding of C1q activates the associated C1r, which becomes an active enzyme that cleaves the proenzyme C1s, generating a serine protease that initiates the classical complement cascade.

(or antigen) is present in the patient’s serum, then the complement is completely utilized and SRBC lysis is minimal. However, if the antibody (or antigen) is not present in the patient’s serum, then the complement binds anti-SRBC antibody and lysis of the SRBCs ensues. Color changes in the solution induced by RBC lysis can be measured spectrophotometrically (Figure 6.10).

**FIGURE 6.10** Complement fixation assay. Left: Antigen is present in the patient’s serum, then the complement is completely utilized and SRBC lysis is minimal. Right: Antigen is not present in the patient’s serum, then the complement binds anti-SRBC antibody and lysis of the SRBCs ensues. SRBC, sheep red blood cells.

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AGGLUTINATION

Agglutination tests are based on the presence of agglutinating antibodies that can react with specific antigens to form visible clumps. When antibodies are mixed with their corresponding antigens on the surface of large, easily sedimented particles such as animal cells, erythrocytes, or bacteria, the antibodies cross-link the particles, forming visible clumps (Janeway et al. 2001). This reaction is termed agglutination. Agglutination is a serological reaction and is very similar to the precipitation reaction. Both reactions are highly specific because they depend on the specific antibody and antigen pair. The main difference between these two reactions is the size of antigens. For precipitation, antigens are soluble molecules, and for agglutination, antigens are large, easily sedimented particles. Agglutination is more sensitive than a precipitation reaction because it takes a lot of more soluble antigens and antibody molecules to form a visible precipitation. To make the detection of soluble antigen and antibody reaction more sensitive, a precipitation reaction can be transformed into an agglutination reaction by attaching soluble antigens to large, inert carriers, such as erythrocytes or latex beads (Van Oss 2000).

Agglutination reactions have many applications in clinical medicine that can be used to type blood cells for transfusion, to identify bacterial cultures, and to detect the presence and relative amount of specific antibody in a patient’s serum. For example, agglutination of antibody-coated latex beads has become a popular commercial method for the rapid diagnosis of various conditions such as pregnancy and streptococcal infections.

ENZYME IMMUNOASSAY (EI)

Methods of using the specific reactivity of antibody with antigen to reveal the presence of antibodies in sera or to identify antigens in tissues and cells differ according to the mechanisms by which the antigen–antibody interaction is revealed. Agglutination and precipitation are classical examples of gross effects produced by antigen–antibody interaction that are more or less immediately visible to the naked eye. Other methods exploit secondary devices as a means of converting antigen–antibody interaction to observable forms. One such device is to label one of the reactants, antigen or antibody, and to use it as a topographical tracer.

Enzyme immunoassay (EIA) is one of such methods that label antigen or antibody with enzyme. The most representative form of EIA is the enzyme-linked immunosorbent assay (ELISA) in which bound antigen or antibody is detected by a linked enzyme that converts a colorless substrate into a colored product (Figure 6.11).

For ELISA, an enzyme is linked chemically to the antibody. The labeled antibody is allowed to bind to the unlabeled antigen, under conditions where nonspecific adsorption is blocked, and any unbound antibody and other proteins are washed away. Binding is detected by a reaction that converts a colorless substrate into a colored reaction product. The color change can be read directly in the reaction tray, making data collection very easy, and ELISA also avoids the hazards of radioactivity. This makes ELISA the preferred method for most direct-binding assays (Plested et al. 2003).
The principle of the enzyme-linked immunosorbent assay (ELISA). To detect antigen A, purified antibody specific for antigen A is linked chemically to an enzyme. The samples to be tested are coated onto the surface of plastic wells. The labeled antibody is then added to the wells under conditions where nonspecific binding is prevented, so that only the labeled antibody binding to antigen A was retained on the surface. Unbound labeled antibody is removed from all wells by washing, and bound antibody is detected by an enzyme-dependent color-change reaction.

**Reagents:**
- **Y**: Ab specific for hormone (coating the filter)
- ***: Unknown sample with hormone
- **: Add anti-A antibody covalently linked to enzyme
- **: Wash away unbound antibody
- **: Enzyme makes colored product from added colorless substrate

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<tr>
<th>Reagents:</th>
<th>POSITIVE SAMPLE</th>
<th>NEGATIVE SAMPLE</th>
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<td>Y: Ab specific for hormone (coating the filter)</td>
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<td>*: Unknown sample with hormone</td>
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<td>**: Add anti-A antibody covalently linked to enzyme</td>
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**Procedure:**
Measure radioactivity in a gamma counter

**Result:**
Amount of radioactivity is inversely proportional to the concentration of hormone in the sample.

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ELISA can also be carried out with unlabeled antibody stuck to the plates and a second labeled antibody added. Rather than the antigen being directly attached to a plastic plate, antigen-specific antibodies are bound to the plate. These are able to bind antigen with high affinity, and thus concentrate it on the surface of the plate, even with antigens that are present in very low concentrations in the initial mixture. A separate labeled antibody that recognizes a different epitope to the immobilized first antibody is then used to detect the bound antigen. This is a modification of ELISA known as “sandwich ELISA” which can be used to detect secreted products such as cytokines (Itoh and Suzuki 2002).

Whittier et al. (19XX) used sandwich ELISA to measure the amount of protein S in patients’ plasma. Monoclonal antibody to human free protein S is coated as a capture antibody to the bottom and sides of microplates of a polystyrene multiple-well plate (96-wells/plate). The wells are stabilized, and noncoated areas of the plastic are blocked to decrease nonspecific binding. Diluted patient plasma is incubated in the wells, allowing patient free protein S to bind specifically to the monoclonal capture antibody. The detection antibody, a solution of polyclonal antihuman protein S antibody conjugated to an enzyme is added to quantitate patient free protein S that is bound to the wells. After incubation, any unbound enzyme-conjugated antibody is washed from the wells. The degree of conjugate binding is measured by adding a chromogenic substrate system (tetramethylbenzidine and hydrogen peroxide) resulting in a soluble colored product. Color development (absorbance) is measured with a spectrophotometer, in optical density units (ODs). Patient ODs are used to determine free protein S antigen levels in relative percent concentrations from a reference curve produced by testing multiple dilutions of assayed reference plasma.

ELISA does not allow one to measure directly the amount of antigen or antibody in a sample of unknown composition, as it depends on the binding of a pure labeled antigen or antibody (Reen 1994). One way to solve this problem is to use a competitive inhibition assay. In this type of assay, the presence and amount of a particular antigen in an unknown sample is determined by its ability to compete with a labeled reference antigen for binding to an antibody attached to a plastic well. A standard curve is first constructed by adding varying amounts of a known, unlabeled standard preparation; the assay can then measure the amount of antigen in unknown samples by comparison with the standard. The competitive binding assay can also be used for measuring antibody in a sample of unknown composition by attaching the appropriate antigen to the plate and measuring the ability of the test sample to inhibit the binding of a labeled specific antibody.

**Radio Immunoassay**

Another method studying antigen-antibody interaction by labeled reactant is radioimmunoassay (RIA) in which antigen or antibody is labeled with radioactivity. The basic principle of a radioimmunoassay (RIA) is the use of radiolabeled Abs or Ags to detect Ag:Ab reactions (Goldberg and Djavadi-Ohaniance 1993). The Abs or Ags are labeled with the $^{125}$I (iodine-125) isotope, and the presence of Ag:Ab reactions is detected using a gamma counter. The unlabeled component, which in this case
would be antigen, is attached to a solid support, such as the wells of a plastic multiwell plate, which will adsorb a certain amount of any protein. The labeled antibody is allowed to bind to the unlabeled antigen, under conditions where non-specific adsorption is blocked, and any unbound antibody and other proteins are washed away. Antibody binding in RIA is measured directly in terms of the amount of radioactivity retained by the coated wells. Labeled anti-immunoglobulin antibodies can also be used in RIA to detect binding of unlabeled antibody to unlabeled antigen-coated plates. In this case, the labeled anti-immunoglobulin antibody is used in what is termed a “second layer.” The use of such a second layer also amplifies the signal, as at least two molecules of the labeled anti-immunoglobulin antibody are able to bind to each unlabeled antibody.

RIAs are highly sensitive and quantitative, capable of detecting small amounts of Ag or Ab. As a result, they are often used to measure the quantities of hormones or drugs present in a patient’s serum. In this case, RIAs are performed in a manner similar to the competitive ELISA. The presence of the hormone in the serum sample inhibits binding of the radiolabeled hormone. Thus, the amount of radioactivity present in the test is inversely proportional to the amount of hormone in the serum sample. A standard curve using increasing amounts of known concentrations of the hormone is used to determine the quantity in the sample.

Another use of the RIA is to measure the quantities of serum IgE antibodies specific for various allergens in a patient’s serum, in which case it is called a Radio Allergo Sorbent Test (RAST) (Nalebuff 1985). In this case, the test is performed similar to an ELISA for Ab, using radiolabeled antiglobulins specific for IgE, rather than enzyme-labeled antiglobulins. However, this test has been almost completely replaced by ELISA.

Because of the requirement to use radioactive substances, RIAs are frequently being replaced by other immunologic assays, such as ELISA and fluorescence polarization immunoassays (FPIA) (Niemann et al. 1985). These have similar degrees of sensitivity. FPIAs are highly quantitative, as are RIAs, and ELISAs can be designed to be quantitative.

REFERENCES

Commonly Used Analytical Techniques for Biotechnology Products


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