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Electrophoresis

in biochemistry

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(*figure graphics by* Richard Buchal)

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The term electrophoresis means any experimental technique that is based on **movement of charged particles (ions, molecules, macromolecules) in electric field in liquid medium**. Any electrically charged particle dissolved in aqueous solution, when placed to a constant electric field, will start to migrate towards the electrode bearing the opposite charge; the speed of the particle movement will be directly proportional to the applied voltage and particle charge, but inversely proportional to the particle size. Any molecules that differ in size and/or charge can be separated from each other in this way. The electrophoretic analysis can in principle be applied to any particles that are charged under given experimental condition, such as small cations or anions, organic acids, amino acids, peptides, saccharides, lipids, proteins, nucleotides, nucleic acids, even the whole subcellular particles or the whole cells. In practice, however, the by far commonest subjects of electrophoretic separation are **proteins** and **nucleic acids**.

General features of electrophoresis

The very basic prerequisite of electrophoretic separation is presence of **charge** on the separated macromolecules. Phosphate groups in structure of DNA and RNA quite reliably give these substances nature of polyanions, which in neutral or alkaline pH migrate to the positively charged electrode (anode). In case of proteins this issue is somewhat more complex, because proteins are **ampholytes**, i.e. they bear both positive and negative groups, and the pH of the medium (electrophoretic buffer) determines whether the resulting net charge of separated protein is positive or negative (Fig. 1). Majority of natural animal proteins has **isoelectric point** in weakly acidic range, pI=5-6. Therefore, in mildly **alkaline electrophoretic buffer (pH about 8.5)**, which is used by far the most often, proteins are polyanions as well, and hence move from the cathode (–) to the anode (+).

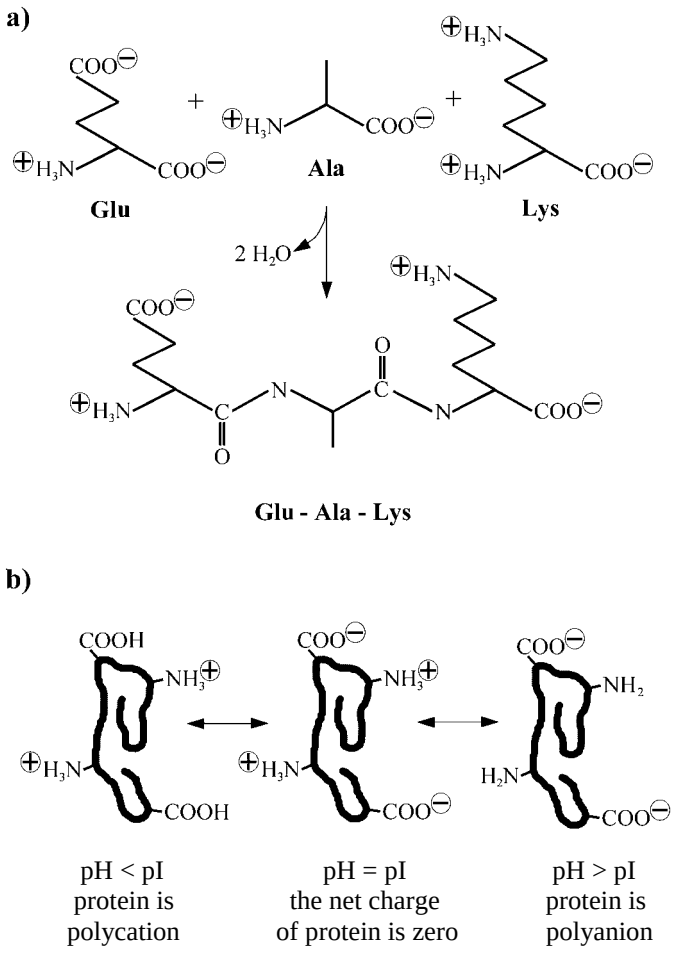


Fig. 1: Charge of proteins.
a) Distribution and number of charges in a simple tripeptide Glu-Ala-Lys in comparison to the original amino acids: The peptidic bond itself is uncharged and in its formation the original charges of carboxyls and α-amino groups disappear. The charge of a polypeptidic chain consists of contributions from the N-terminal amino group, C-terminal carboxyl, and ionized groups in the side chains of certain amino acid residues, mostly carboxyls of Asp, Glu, and amino- or imino-groups of Arg, Lys and His.

b) Typical protein contains many acidic as well as basic ionizable groups, each with its own dissociation constant making protonation dependent on pH of the medium. In general carboxyl groups are protonated and hence uncharged in acidic medium, while they bear negative charges at neutral or alkaline pH. In contrast, the amino groups are protonated and hence positively charged in neutral or acidic medium, while in alkali they lose protons as well as charges. The presence of many ionizable groups causes the net charge of a protein being a continuous function of pH. Isoelectric point (pI) is a pH value at which the intensities of positive and negative charges on a protein are equal and the net protein charge is zero. The value of pI is determined by protein primary structure (sequence) and therefore is different for different proteins.

Separation of serum proteins by electrophoresis was first attempted by Tiselius in 1937. In his experiment the proteins moved to the oppositely charged electrode in **free solution**. Such **'free electrophoresis'**, however, suffers from one important disadvantage: passage of electric current produces heat that brings about convection flow of the liquid medium, which, in turn, disturbs the zones of separated proteins. Therefore, nowadays electrophoresis is usually performed in some **support**, which can be paper, cellulose acetate, or **gel** made of starch, **agarose** or **polyacrylamide**. The latter two materials are used by far most often. The support – gel – can be envisaged as a three-dimensional structure of open pores filled with a liquid; this space structure prevents convection flows of electrophoretic buffer during separation. Simultaneously, however, size of the gel pores, which especially in polyacrylamide can be easily controlled, represents another important factor affecting result of an electrophoretic separation. If the pores are large enough to allow free movement of macromolecules, the separation of proteins will be the same as in free solution, i.e. according to the charge/size ratio (more exactly stated: according to the **surface charge density**). On the other hand, a thicker gel with small pores will present a mechanical obstacle to the movement of proteins, i.e., the gel will act as a **'molecular sieve'**, slowing down the protein movement the more the bigger the proteins are. As will be shown below, the protein electrophoresis can be modified to achieve separation based purely and exclusively on the protein charge, or purely on the protein size. Nucleic acids, whose charge density does not depend on their size (every other nucleotide bears the same charge), can actually be separated only according to their size, i.e., in gels dense enough to act as molecular sieves.

Another important attribute of particular electrophoretic techniques is their **resolution**, which can be in a simplified way defined as a **number of protein bands/spots** obtainable by separation of a complex mixture. In a simple electrophoretic setup, where proteins migrate to the opposite electrode in some **support**, separation of protein zones is accompanied by their broadening because of simultaneously occurring **diffusion**. One way how to overcome this resolution limit is simply to fasten the separation, i.e., to apply **higher voltage**. In general, the speed of charged particle migration in an electric field is directly proportional to the field intensity (in V/cm), charge of the particle, and temperature of the medium, but inversely proportional to the particle size and resistance of the medium. Higher voltage results in faster migration of particles; simultaneously, however, for the same medium resistance according to the Ohm's law the higher voltage must result in higher electric current, thus (markedly) higher generation of heat. Notably, this **Joule's heat** resulting from passage of electric current rises linearly with the medium resistivity, but exponentially (second order) with the intensity of passing current. Another possibility how to improve resolution is to increase **ionic strength** of the electrophoretic medium: better resolution of protein bands in this case is explained as a consequence of larger ionic clouds surrounding the separating macromolecules. But, on the other hand, a higher ionic strength means higher conductivity (lower resistance) of the medium, and hence again for a given voltage more current passes and more heat is generated. Finally, the ultimate practical limit of the resolution power of simple protein electrophoresis becomes ability to provide the whole system with an efficient **cooling**. However, there are also electrophoretic techniques where certain specific physico-chemical conditions help to **focus** the protein bands during their separation, and high resolution is thus achieved by other means.

Next, the principles of basic electrophoretic techniques of (mostly) proteins will be briefly introduced, together with their applications and subsequent procedures (more about electrophoresis of DNA can be found in the text on molecular biology methods).

Native protein electrophoresis

This is a basic arrangement of electrophoresis, in which the proteins retain their original (native) conformation and oligomeric structure (subunit composition), charge and biologic activity, and in mildly alkaline buffer migrate to the positively charged electrode. **Agarose** is probably the most commonly used support for this type of analysis, although similar results can also be obtained with cellulose acetate and thin polyacrylamide (as on Fig. 3). The agarose gel is customary also in basic electrophoresis of nucleic acids, which in this medium separate according to the size. For proteins, however, the pores in agarose are too large for molecular sieving – **protein separation takes places according to their surface charge density**. Proteins that differ in size, but not in charge density,

migrate with the same speed and cannot be resolved (e.g. albumin dimer and monomer). The resolution is limited because there are **no further focusing mechanisms**. Normal **human serum** classically separates to **5 fractions: albumin, α1-, α2-, β- and γ-globulin.**

The protein separation in agarose sometimes displays an interesting, although rather unwanted phenomenon of **electroendoosmosis**, which manifests as movement of the slowest fraction (γ-globulins) *against the direction* of its expected electrophoretic mobility, i.e., from the start towards the cathode (negative electrode). The reason for this phenomenon is presence of negatively charged groups in the agarose gel, around which a cloud of partially mobile positive ions from the electrophoretic buffer forms. Once voltage is applied, these positively charged ions start to move towards cathode and drag the most slowly migrating proteins with them. In purified agarose lacking the charged groups and in polyacrylamide the electroendosmosis does not occur.

Native **electrophoresis of human serum proteins** is one of the **basic biochemical examinations**, routinely performed in the clinical chemistry laboratories. It is also used for analysis of isoenzymes, spectra of serum lipoproteins, hemoglobin variants etc.

Polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE)

In this technique the electrophoretic separation of proteins is preceded by their **denaturation** (Fig. 2a), by treatment with a strong anionic detergent **sodium dodecylsulfate** (SDS, $CH_3(CH_2)_{11}OSO_3Na$). All proteins indiscriminately bind SDS, although for full denaturation the sample needs to be **heated**, typically 100 ˚C for 10 minutes, and simultaneously **cleavage (reduction) of disulfidic bridges** in and among the chains must be enabled by addition of mercaptoethanol or dithiothreitol. A protein treated in this way loses its native conformation and biologic activity; in addition, the amount of **bound SDS anions** is so high that the original charge of the protein becomes insignificant. All polypeptidic chains then display the **same charge density**, and subsequent electrophoresis on polyacrylamide in the presence of SDS (**SDS-PAGE**) separates the proteins **exclusively according to their size**. If suitable standards are included, this technique can be employed for estimation of **molecular weight** of a studied polypeptidic chain.

SDS-PAGE is usually performed as **discontinuous**, which means that the polyacrylamide gel is cast in two layers: the sample proteins first enter the thinner **concentrating gel**, where they focus to a narrow zone; subsequently in the second denser **separating gel** and higher pH the separation according to size takes place (see Fig. 2b for detailed explanation). In this way a considerable resolution power is achieved, for example serum can be resolved to few dozens of proteins bands (Fig. 3).

This technique is nowadays probably **the most widely used** approach to electrophoretic analysis of proteins in research labs, favored for the stability of denatured samples, relative feasibility, and high resolution. It finds usage also in some clinical chemistry analyses of protein spectra of body fluids, for example in examination of **proteinuria** (pathologic presence of proteins in the urine).

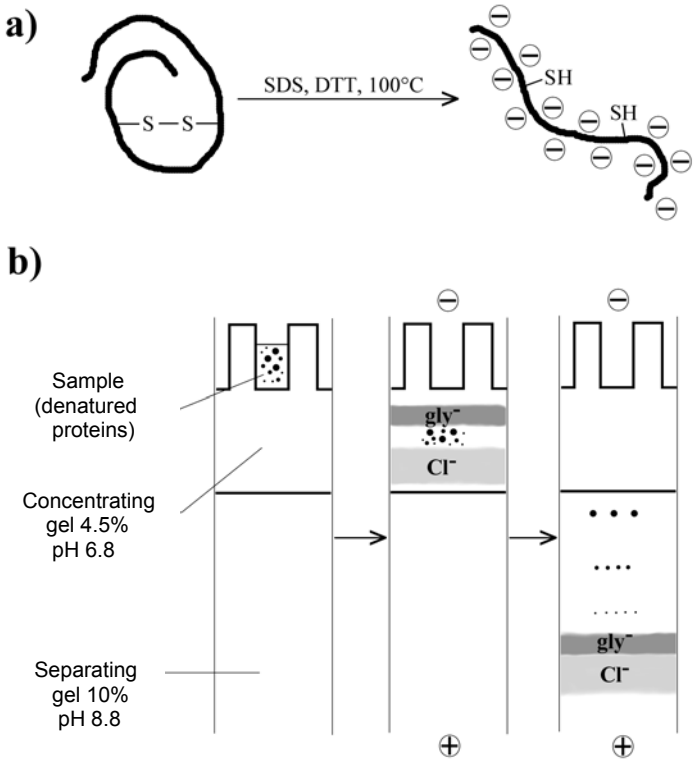
Gradient PAGE

The size of pores in polyacrylamide gel can be easily controlled by the composition and monomer concentration in the polymerisation mixture. It is even possible to prepare a gel in which **pore size gradually decreases**, i.e. the density of gel continuously increases, towards the anode. Proteins migrating in such gel during electrophoresis encounter ever increasing resistance of the gel support, their migration is progressively retarded and finally ceases. Size of the protein matters (smaller protein migrates farther), and so this technique enables **separation of native proteins or protein complexes according to their size**. Resolution is high because the protein bands tend to focus during their separation: the protein molecules that are ‘behind’ are still in a thinner gel, hence move faster and tend to ‘catch up’ the protein molecules that are more ‘ahead’ and so more retarded by the gel resistance.

Fig. 2: Principle of discontinuous SDS-PAGE.

a) Denaturation of the sample proteins by boiling with sodium dodecylsulfate (SDS, binds to polypeptidic chain, disrupts protein conformation and brings a strong negative charge) together with dithiothreitol (DTT, reduces and hence cleaves disulfidic bonds).

b) Mechanism of focusing of protein bands. Electrophoretic buffer contains a single cation ($Tris^+$) and more anions (Cl^- and glycinate). **Middle:** SDS-denatured proteins first enter the thinner concentrating gel, which does not retard migration of proteins. Because of bound SDS all proteins have the same charge density and migrate with the same speed. The buffer anions move to the anode as well, and at pH 6.8 the proteins are squeezed between the zones of chloride and glycinate ions, which focus them to a single narrow zone. **Right:** following entry to the separating gel the higher pH increases ionization of glycinate ions making them moving faster than the proteins. The denser gel simultaneously retards movements of proteins in dependence on the protein size due to molecular sieving effect. The proteins start to separate according to their size.



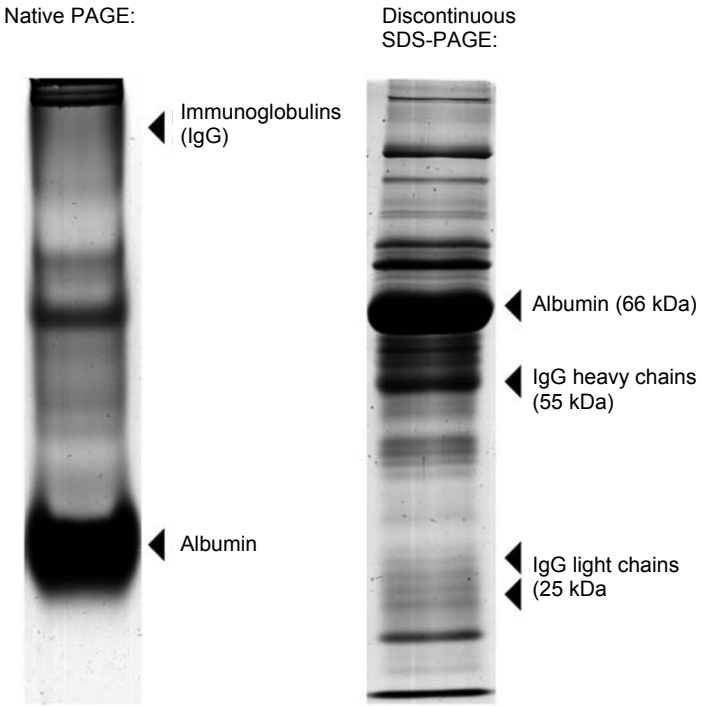


Fig. 3: Normal rabbit serum subjected to native electrophoresis in polyacrylamide (left) and the SDS-PAGE (right). Notice the substantially higher resolution power of the SDS-PAGE. Albumin (MW cca 66,000) as the most abundant serum protein consisting of a single polypeptide is easily identified on both electrophoreograms. In contrast, for the immunoglobulin IgG, a tetramer composed from two heavy and two light chains joined by disulfidic bonds, the results of both electrophoreses are different: while under native condition it migrates as the slowest γ-globulin fraction, on SDS-PAGE its heavy chains (MW cca 55,000) and light chains (MW cca 25,000) appear separately.

Isoelectric focusing

The technique called isoelectric focusing (IEF) separates proteins purely and **exclusively according to their charge**, and allows determination of **isoelectric point** of a protein. This is achieved by electrophoretic separation of proteins in **gradient of pH**, resulting from a **complex mixture (50-100) of organic ampholytes** (amphoteric polyaminocarboxylic acids), each with different isoelectric point. If voltage is applied, the ampholytes position in the gel according to their isoelectric points, which produces the continuous pH gradient. The sample proteins, then, in this environment migrate to the anode or cathode until each protein finds a place where **the local pH corresponds to its isoelectric point**, and stays there because at pH=pI the net protein charge is zero. The bands display a **tendency to focusing** because once a protein molecule diffuses out from the place where pH=pI, it regains charge and is forced by the electric field to move back to the band center (that is why it is called isoelectric *focusing*). This technique can resolve proteins whose pIs differ by as little as 0.02 pH units.

2D Electrophoresis

In the two-dimensional (2D) electrophoresis a mixture of proteins is first subjected to separation according to their charge by isoelectric focusing, and then in the second dimension according to their size in the presence of SDS (Fig. 4). Combination of two separations, one based on pI and the other one on molecular weight, offers resolution that cannot compare to any of the '1D' techniques – one sample on a single 2D gel can yield *several thousands* of protein spots.

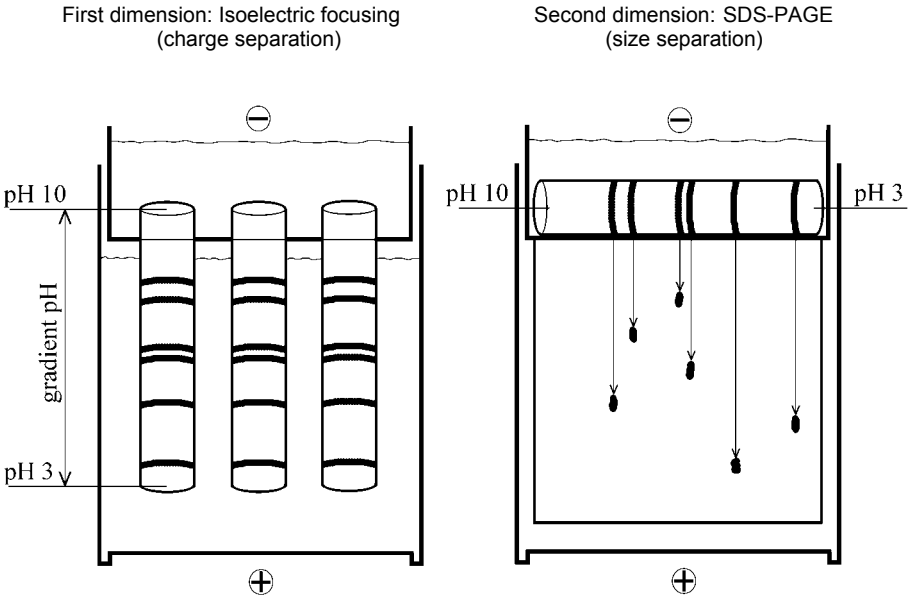


Fig. 4: Principle of 2D electrophoresis. Left: isoelectric focusing in rods of thin polyacrylamide, proteins migrate in pH gradient and make separated zones according to their isoelectric points. **Right:** following the IEF the gel rod with protein bands is equilibrated with SDS and placed upon a slab of a denser polyacrylamide, protein zones from the first dimension now undergo second separation according to the molecular weight of polypeptide chains. (According to materials of Pharmacia Fine Chemicals, Uppsala, Sweden, 1983)

Staining, densitometry and blotting

Gel electrophoresis of proteins or nucleic acids is always followed by a **detection of separated sample components**. For visualization of DNA in agarose gel a fluorescent dye **ethidium bromide** is used. Likewise, all proteins separated by electrophoresis in a gel can be stained with e.g. **Coomassie Brilliant Blue** or **AmidoBlack**. If electrophoreogram of serum proteins is stained for lipids (**Sudan Black**), it provides information about the spectrum of serum lipoproteins. The intensity of particular protein bands can be quantified by means of **densitometry**, which in principle is a kind of photometric analysis – a light beam passes through stained and fixed electrophoreogram, and degree

of its absorption by protein bands is read by a photodetector at the other side. The results of routine electrophoresis of serum proteins in clinical chemistry are usually given with a densitometric evaluation as protein fractions in % of total.

The resolving power of electrophoresis can also be coupled to a **specific detection** of certain DNA sequence by means of **hybridization with a labeled DNA probe**, or detection of a certain protein by means of **interaction with a specific and labeled antibody**. These approaches are extremely powerful and widespread. However, it is usually difficult to perform these detection reactions with macromolecules entrapped within a three-dimensional mesh of the gel support. Therefore, electrophoresis is very often followed by **blotting**, which means **transfer of DNA fragments or proteins from gel onto surface of a blotting membrane**, where the macromolecules of interest are more accessible to various specific detection procedures. The blotting membrane can be made of nitrocellulose, nylon, or PVDF (polyvinylidene fluoride). The transfer may be driven just by capillary force (rise of liquid through gel pores); nowadays vacuum or transversally applied electric field is often used. The technique of blotting was invented by Professor Edward Southern in 1975 for electrophoresed fragments of DNA. In his honor the blotting of DNA has been called **Southern blotting**. Later on, further applications of the blotting technique were given names according to the other cardinal points: blotting of RNA is called **Northern blotting**, while modification of the technique for proteins is known as **Western blotting**.

Capillary electrophoresis

All the examples of gel electrophoresis explained above are performed in rods or (much more often) slabs of cast gel. In capillary electrophoresis the technical arrangement is different: the separation takes place in very thin (internal diameter 10-100 μm) and fairly long (20-200 cm) **capillary**, which extends with its ends to the chambers containing electrophoretic buffer and the electrodes. The actual electrophoretic separation can be based on any of the aforementioned principles, i.e., it can proceed as free or gel electrophoresis, isoelectric focusing etc. A portion of capillary typically serves as window for detection of separating components that can be based on **photometry** or **laser-excited fluorescence**. No permanent electrophoreogram is obtained; rather, the whole arrangement resembles chromatographic analysers.

The capillary electrophoresis offers two critical advantages. The first one is possibility of **efficient removal of electric current-generated heat** just because of small diameter of the capillary. Consequently, high voltage can be applied that shortens time of the whole analysis (sometimes even less than 1 minute per sample). The thin capillary also solves the problem of convection flow in free electrophoresis. The other big advantage is **easy automation**. The routine electrophoresis of serum proteins can be completely automated in this way. Another good example is the **Sanger's technique of DNA sequencing**, also based on electrophoresis. Replacement of the original laborious hand-made cast sequencing gels with the capillary electrophoresis was one of the critical advances that enabled development of **efficient automated sequencers**, necessary for the formidable task of human genome sequencing.

Proteomics

Once the human and other genomes have been successfully sequenced, the next major challenge in the biomedical research becomes identification and characterization of all the proteins that constitute human body or bodies of other model organisms – i.e., the whole **proteomes**. The research brand dealing with this task is called **proteomics**. Technically, the **proteomics is based on 2D-electrophoresis**, whose tremendous resolution power enables separation of even the most complex sample to thousands of spots – individual protein species. The next step is **cutting each spot** from the gel and **identification of each protein**. A typical approach employs **limited digestion** of the protein by a **specific protease**, which cleaves only certain sequences (e.g., trypsin cleaves behind Arg and Lys unless the next residue is Pro), and the resulting **mixture of peptidic fragments** is subjected to analysis by **mass spectrometry**, able to measure the peptide molecular weight with precision to fractions of Dalton. Comparison of the measured fragment masses (the peptide mass fingerprint) with the ones predicted from the virtual cleavage of known protein sequences in the **internet databases** can lead to identification of protein.