Kód předmětu: Bi8980

MASARYKOVA UNIVERZITA

Protein expression and purification

X. Analytical methods used to study proteins

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Tento projekt je spolufinancován Evropským sociálním fondem a státním rozpočtem České republiky.

EVROPSKÁ UNIE

MINISTERSTVO ŠKOLSTVÍ, MLÁDEŽE A TĚLOVÝCHOVY

OP Vzdělávání pro konkurenceschopnost

INVESTICE DO ROZVOJE VZDĚLÁVÁNÍ

The most commonly used analytical techniques

- Determination of protein concentration
- Electrophoresis
- Enzyme assays
- Binding assays

The key characteristics of the protein:

- concentration
- purity
- relative molecular mass
- enzymatic activity
- binding properties

1. Determination of protein concentration

- *Colorimetric assays* (complex protein mixtures: lysates, intermediate steps in a purification):
  - Dye binding (Bradford)
  - Bicinchoninic acid (Smith)
  - Biuret
  - Lowry

- *Absorbance assays* (purified proteins):
  - absorbance at 280 nm
  - absorbance at 205 nm

1.1. Colorimetric assays – general approach

- Comparative, i.e. based on the use of a reference (standard) protein
  - Preparation of stock solution of reference protein (BSA, IgG)
  - Construction of calibration curve for reference protein
  - Determination of unknown protein using the calibration curve

- Work within the linear range of an assay, that is, where absorbance is directly proportional to concentration.

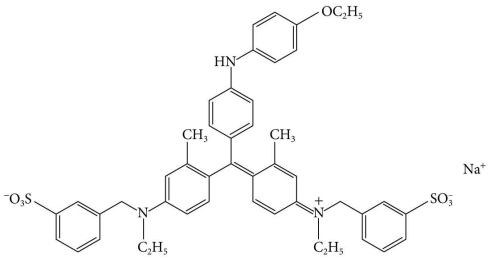
- When samples are so concentrated that you cannot pipette a small enough amount accurately, you may have to conduct serial dilutions.

1.1.1. Dye binding assay – Bradford

Principle:

The absorbance maximum (λ_{max}) for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs.

- λ_{max} 465 nm: reddish/brown (protonated form)
- λ_{max} 595-620 nm: blue (deprotonated form)



- dependent on the amino acid composition – the dye reagent reacts with side chain NH_3^+ groups → primarily with Arg and less so with Lys, His, Tyr, Trp, and Phe
- is less accurate for basic or acidic proteins
- free amino acids, peptides and low molecular weight proteins (<3,000 Da) do not produce color with Coomassie dye reagents

Bradford, M. M. (1976): Anal. Biochem. **72**: 248-254

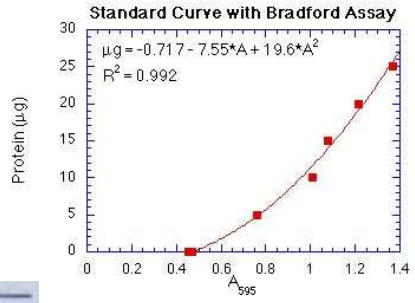
1.1.1. Dye binding assay - Bradford

Advantages

- fast and inexpensive
- highly specific for protein
- very sensitive: 1–25 µg
- Compatible with a wide range of substances (major interfering agents: detergents (SDS))

Disadvantages

- assay is linear over a short range
- response to different proteins can vary widely; choice of standard is very important



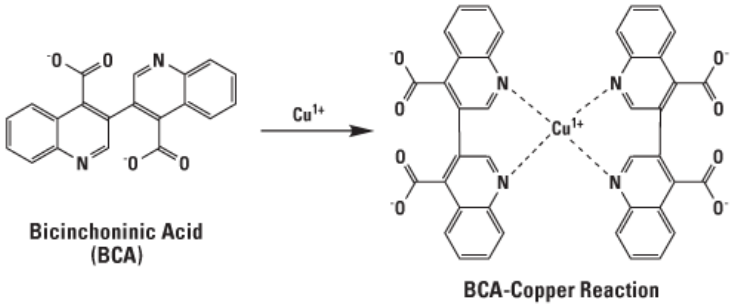
Protein used in each assay (µg)	A_{595}			
	BSA	Lysozyme	Trypsin	Ovalbumin
0	0	0	0	0
5	0.23	0.22	0.08	0.12
10	0.42	0.43	0.14	0.22
15	0.59	0.60	0.19	0.30
20	0.75	0.76	0.24	0.38
25	0.89	0.90	0.29	0.45

Modification:Thermo Scientific Pierce 660 nm Protein Assay – compatible with most detergents and produces a more linear response curve

1.1.2. Bicinchoninic acid (BCA or Smith) assay

Principle

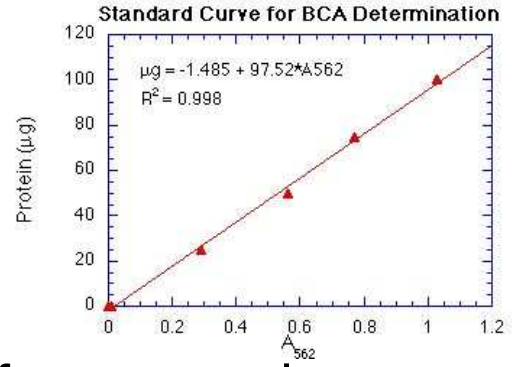
Under alkaline conditions, Cu^{2+} present in the BCA reagent forms complexes with peptide bonds and becomes reduced to Cu^{+}. Cu^{+} subsequently interacts with BCA to form a purple BCA-Cu^{+} complex detectable at 562 nm.



green → purple

Smith et al. (1985) *Anal. Biochem.* 150: 76-85.

1.1.2. Bicinchoninic acid (BCA or Smith) assay



Advantages

- very sensitive 0.2–50 μg
- compatible with many detergents
- broad linear working range
- little variation in response between different proteins

Protein used in each assay (μg)	A_{562}		
	BSA	Chymotrypsin	Ovalbumin
0	0	0	0
10	0.57	0.71	0.52
20	1.15	1.42	1.05
30	1.75	2.29	1.68
40	2.28	2.79	2.05
50	2.79	3.24	2.38

Disadvantages

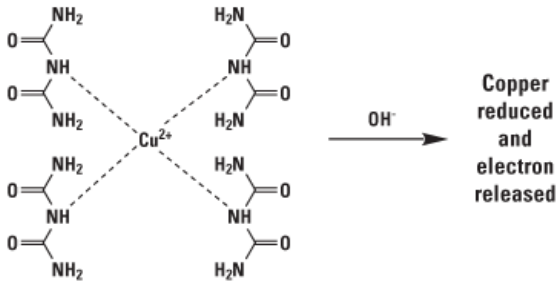
- long incubation time (~1 h), required warming (60°C) → This can be a problem if it is assaying a large number of proteins.
- major interfering agents: glucose, strong acids (EDTA), ammonium sulfate, lipids

1.1.3. Biuret assay

Principle

Under strongly alkaline conditions proteins containing two or more peptide bonds form a purple complex with Cu^{2+} salts in the Biuret reagent (absorption at 540 nm).

Protein used in each assay (mg)	A_{540}		
	BSA	Ovalbumin	Trypsin
0	0	0	0
1.5	0.19	0.20	0.21
3.0	0.37	0.39	0.41
4.5	0.56	0.59	0.62
6.0	0.75	0.79	0.86
7.5	0.97	1.02	1.07



Biuret Reaction

Advantages

- very little variation in response between different proteins
- broad linear working range

Disadvantages

- low sensitivity: 0.5–5 (10) mg
- major interfering agents: ammonium salts

1.1.4. Lowry assay

Principle

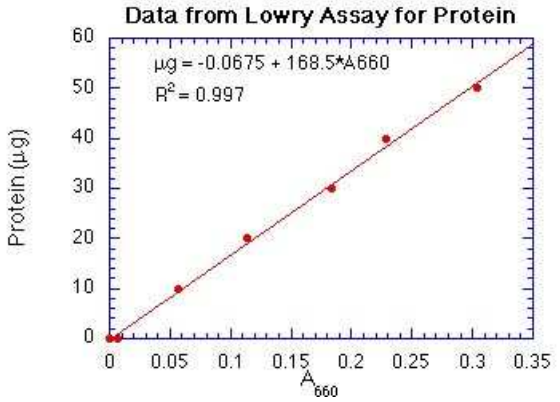
Based on two different reactions:

1. The formation of a Cu^{2+} complex with peptide bonds, forming reduced Cu^{+} in alkaline solutions (biuret reaction).
2. The reduction of Folin-Ciocalteu reagent (phosphomolybdic-tungstic acid) by Cu^{+}, together with side chains of Tyr, Trp, Cys, and His.

The reduced Folin-Ciocalteu reagent is blue → detectable at 750 nm.

Advantages

- sensitive: 5–100 µg
- the standard curve is linear
- very little variation in response between different proteins



Protein used in each assay (µg)	A_{750}		
	BSA	Chymotrypsin	Myoglobin
0	0	0	0
20	0.34	0.38	0.29
40	0.67	0.74	0.56
60	0.99	1.12	0.85
80	1.33	1.47	1.14
100	1.64	1.87	1.44

Lowry et al. (1951) *J. Biol. Chem.* **193**: 265.

1.1.4. Lowry assay

Disadvantages

- many interfering agents: detergents, carbohydrates, glycerol, DTT, tricine, EDTA, 2-mercaptoethanol, Tris, potassium compounds, sulfhydryl compounds, disulfide compounds, magnesium and calcium, Tyr, Trp, Cys, His, and Asp
- takes 40 min
- alkaline copper reagent must be prepared fresh daily

Modification of Lowry

- The Bio-Rad *DC* (detergent compatible) protein assay
- The Bio-Rad *RC DC* protein assay
  - For protein determination in the presence of reducing agents and detergents.
- Hartree-Lowry: Hartree (1972), *Anal. Biochem. 48:* 422-427.
  - This modification makes the assay linear over a larger range than the original assay.
- J.R. Dulley and P.A. Grieve (1975) *Anal. Biochem*. 64: 136.
  - This modification includes 0.5% sodium dodecyl sulfate in the alkaline reagent. This obviates interference from many detergents and helps disperse membranes in the sample.
- A. Bensadoun and D. Weinstein (1976) *Anal. Biochem.* 70: 241.
  - Modification that can be useful when the solution contains interfering contaminants. The proteins in the samples are precipitated by a mixture of sodium deoxycholate and trichloroacetic acid and centrifugation prior to assay. If the contaminants stay in the supernatant they can be removed and the amount of precipitated protein determined.

1.2. Absorbance at 280 and 205

- For pure proteins
- Fast and convenient, no additional reagents or incubations are required.
- No protein standard needs to be prepared.
- Buffer must not absorb at 280/205, or, if it does, an accurate blank must be taken (imidazole, HEPES, MOPS absorb in the far UV).
- Non-aggregated proteins (presence of aggregated proteins leads to light scattering, increasing absorbance values)
- Significant levels of nucleic or oligonucleotide fragments $\rightarrow A_{205}$

A_{280}/A_{260} for proteins ~ 1.7
(for nucleic acids ~ 0.62)

1.2.1. Absorbance at 280 nm

- Radiation absorbance due to Tyr, Trp and, to a lesser extent, Cys
- A_{280} → protein concentration: requires the molar absorbance coefficient (ε) determined from amino acid composition

Denaturated (unfolded) protein:

ε of the protein (280 nm: $M^{-1}cm^{-1}$)=(∑ Tyr x 1280)+(∑ Trp x 5690)+(∑ Cys x 60)
(modified from Gill & Hippel, 1989)

Native (folded) protein:

ε of the protein (280 nm: $M^{-1}cm^{-1}$)=(∑ Tyr x 1490)+(∑ Trp x 5500)+(∑ Cys x 62.50)
(Pace et al., 1995)

[in both equations all Cys form disulfide bonds (cystines), if not: ∑ Cys = 0]

1M solution of protein, of molecular mass X Da, is X mg/ml:

For 1 mg ml^{-1} protein solution: A_{280} = ε of the protein / M_r (Da)

(A_{280} for 1 mg ml^{-1} protein solution, calculated according to equation 1, are available from Protparam (Expasy), Swiss-Prot, TrEMBL)

A_{280} between 0.1 and 1

1.2.2. Absorbance at 205 nm

- Peptide bonds absorb radiation < 240 nm
- For proteins and peptides with low Tyr, Trp
- For proteins with significant nucleic acid contamination
- Protein c (mg mL^{-1}) = $A_{205}/31$
- But more reliable: $A_{205}(1mg\ mL^{-1}) = 27+120\ (A_{280}/A_{205})$ (Scopes, 1974)
  - factor 27: A_{205} of 1mg mL^{-1} protein solution
  - factor 120: empirical value (chosen from studies of variety of standard proteins) to take into account the contribution of Tyr and Trp at 205 nm

2. Electrophoresis methods

- One-dimensional SDS-PAGE (denaturating PAGE)
- Native PAGE (non-denaturating PAGE)
- Isoelectric focusing
- Two-dimensional gel electrophoresis
- Immunoelectrophoresis

2.1. One-dimensional SDS-PAGE

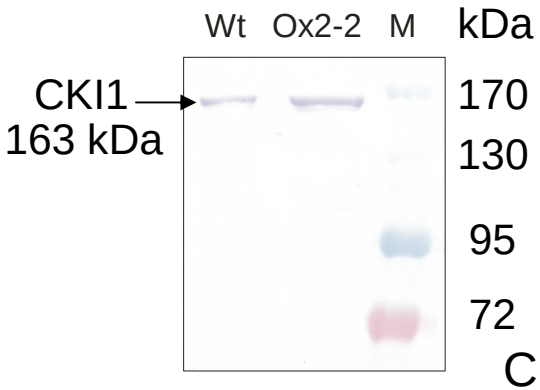
= sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Applications: - estimation of protein subunit molecular mass
- assessment of the protein purity

Proteins are separated according to their size – SDS → constant negative charge: for most proteins

1.4 g of SDS/1 g of protein

!Membrane proteins – gel shift!



CKI1: 125 kDa

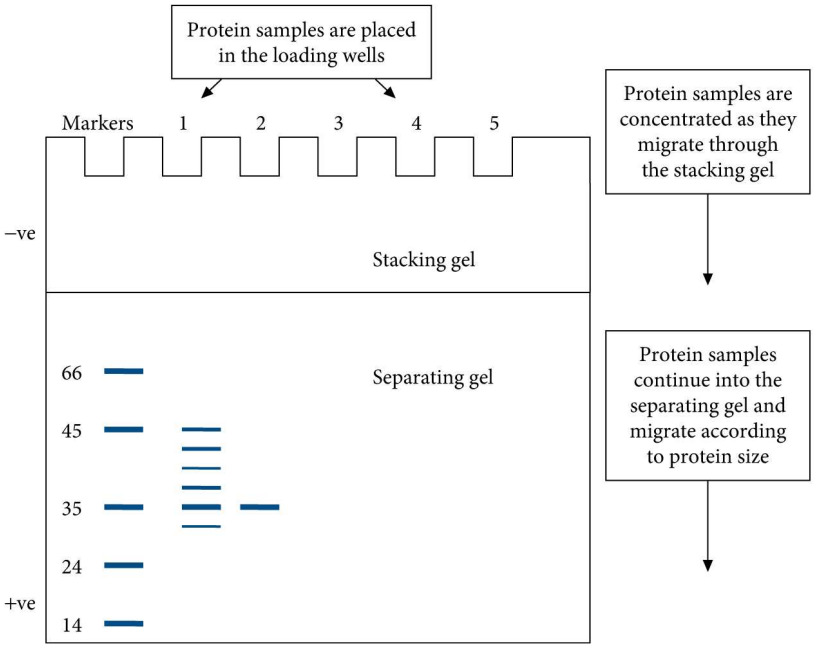
Table 1. Gel shifts of various helical membrane proteins on SDS-PAGE

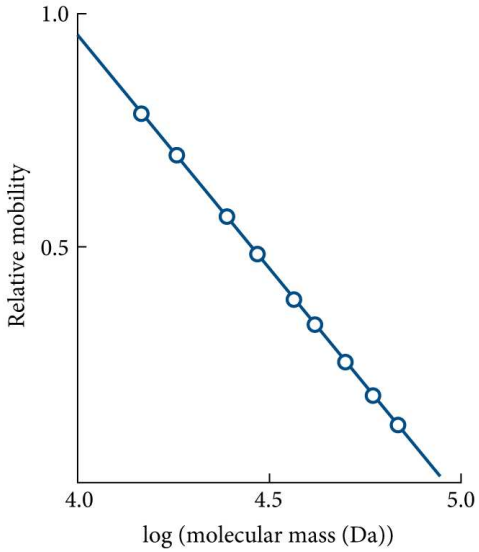
Protein*	MW (kDa) App.	MW (kDa) Formula	Gel shift dMW, %†	Ref.
I. tartaricus F-type ATPase c subunit				41
Undecamer	53	97	−46	
Monomer	6.5	8.8	−26	
Archaeal ammonium transporter Amt-1				42
Trimer	90	134	−33	
Monomer	33	45	−27	
E. coli lactose permease	33	47	−30	43
Vitamin B12 transporter BtuC	26	35	−26	44
H^+/Cl^- exchange transporter ClC	38	51	−25	45
Potassium channel KcsA tetramer	60	76	−21	46
Phospholamban				47
Pentamer	29	30	−17	
Monomer	9	6.1	+48	
Particulate methane monooxygenase				48
pmoA subunit	24	28	−14	
pmoB subunit	47	46	2	
pmoC subunit	22	30	−27	
Glycerol facilitator channel GlpF	29	34	−15	49
E. coli MscS channel				50
Dimer	52	62	−16	
Monomer	27	31	−12	
Multidrug transporter AcrB	100	115	−13	51
Glycerol-3-phosphate transporter GlpT	45	50	−10	52
Spinach aquaporin	31	32	−3	53
Rhomboid family protease GlpG	31	31	0	54
M. tuberculosis MscL channel monomer	20	16	+26	55
β2-adrenergic receptor	62	47	+30	56
M. thermoautotrophicum MthK tetramer	200	149	+34	57

Rath et al., 2009

2.1. One-dimensional SDS-PAGE

Discontinuous system – Laemmli (1970)





Effective separation range of uniform polyacrylamide gels					
% Acrlyamide	5	7.5	10	12	15
Separation range (kDa)	57-212	36-94	20-80	12-60	10-43

2.1. One-dimensional SDS-PAGE

Experimental design

Scale of analysis

- small format (8 x 10 cm): rapid separation
- large format (20 x 16 cm): when high resolution is required

Complexity of the sample

- sample of a few proteins: 1 µg/lane (small format, CB staining)
- complex sample: 20 µg/lane (small format, CB staining)

Suitable detection methods

- Coomassie Brilliant Blue: 0.5 ug
- silver: 0.1ug
- immunodetection: ng

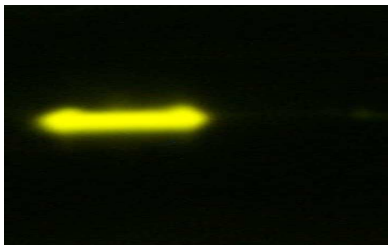
Percentage of polyacrylamide gel

Type of marker

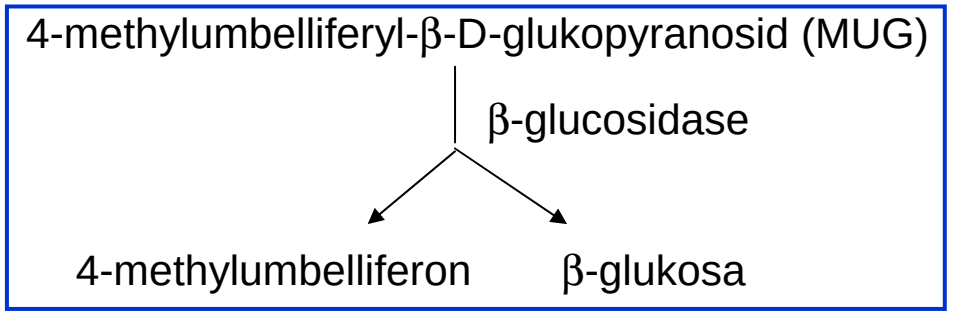
- mobility greater than and less than protein of interest
- mobility in a linear fashion relative to log (molecular mass)

2.2. Native electrophoresis

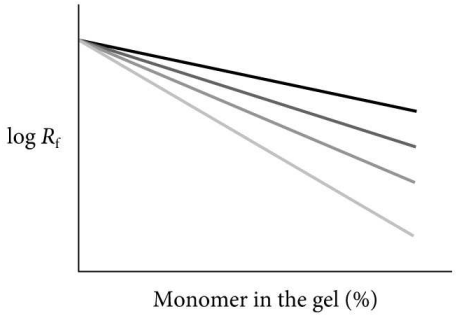
- Proteins are separated by their size, charge and shape
- Non-denaturing conditions → proteins remain stable and in their native form → structure and function retention



β-glucosidase



- Determination of molecular mass, charge, subunit composition (quaternary structure)



  - Calibration proteins with similar mass, different charge → protein charge determination
  - Calibration proteins with similar charge, different mass → protein mass determination → Ferguson plot (evaluated using program ElphoFit)

2.3. Isoelectric focusing (IEF)

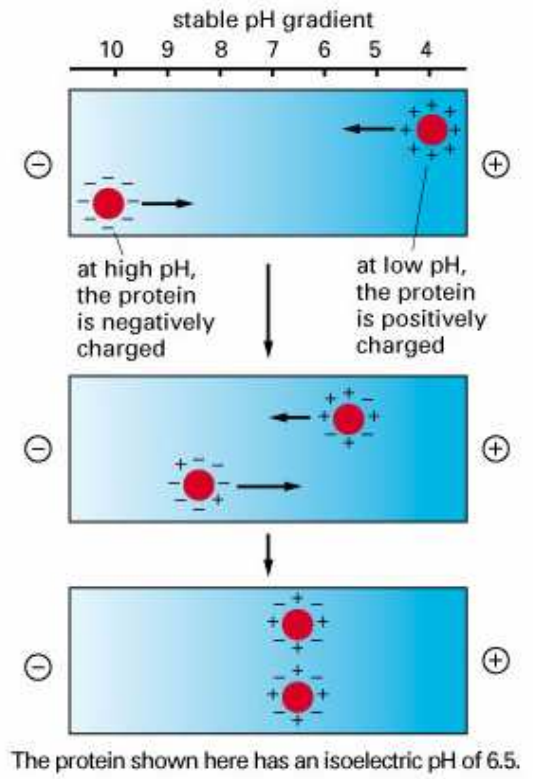
It is a high-resolution method which separates proteins according to their pI.

Proteins that are structurally very similar and have pI that differ by as little as 0.1 pH units can be resolved by IEF.

Proteins carrying a net positive charge migrate towards the cathode and lose or gain protons (if below or above, respectively) until they reach pH=pI
Proteins carrying a net negative charge migrate towards the anode and lose or gain protons (if below or above, respectively)

Success relies on the ability to resolve the proteins in the sample → optimization of pH gradient range and the size of electric field

Proteins in the sample must be soluble and free from salts



The protein shown here has an isoelectric pH of 6.5.

http://www.bio.miami.edu/~cmallery/255/255tech/ecbxp4x4_focus.jpg

2.4. Two-dimensional gel electrophoresis

Proteins are separated in two dimensions:

IEF → SDS-PAGE

Applications:

- analysis of complicated mixtures
- quantification of each protein within a complex mixture
- identification of changes in protein expression pattern
- characterization of subtle post-translational modifications

Experimental design

- loading of the optimal amount of sample to allow separation and detection of proteins which are abundant as well as those which are less abundant
- selection of a suitable pH range for the first dimension
- selection of an appropriate acrylamide gel for optimal separation in the second dimension
- staining: silver (0.1 µg)

  Fluorescent detection: Sypro Ruby (1–2 ng), Sypro Rose (1–2 ng), Flamingo

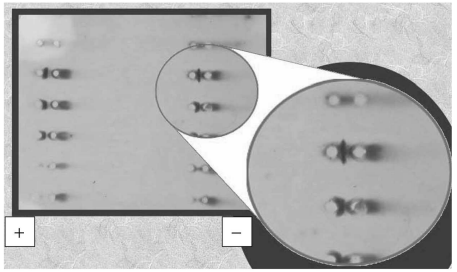
  Radioactive detection

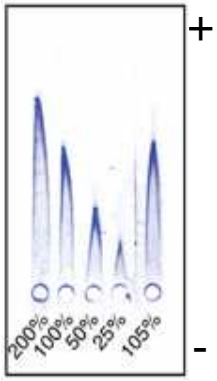
2.5. Immunoelectrophoresis

Antigens (Ag) and antibodies (Ab) are separated by migration through an electric field

Specific Ag-Ab interactions → formation of precipitates (precipitin)

In low concentration agarose gels (1%), Ag migrate towards the anode, Ab move towards the cathode.



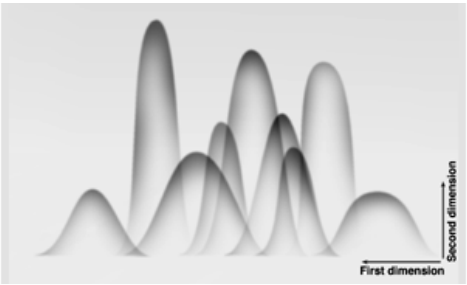


Techniques:

- Cross-over electrophoresis
  Ab and Ag are added to the wells.

- Quantitative immunoelectrophoresis (rocket immunoelectrophoresis)
  Ab is added to the agarose during gel preparation, Ag in the wells.

- Two-dimensional immunoelectrophoresis
  Ag are separated by native elfo and subsequent gel is applied to the top of an agarose gel impregnated with Ab. Application of electric field → migration of Ag into the agarose gel → precipitin



3. Enzyme assays

Enzyme assays = methods by which we can obtain quantitative information about the catalytic activity, including parameters such as the Michaelis constant (K_m), maximum (or limiting) velocity (V_{max}, k_{cat}), and the catalytic efficiency k_{cat}/K_m.

By studying the activity under different conditions *in vitro*, we can evaluate how the enzyme works under conditions close to those operating *in vivo* → this may help in the design of specific inhibitors (simvastatin = HMG-CoA reductase inhibitor treats high blood cholesterol levels).

The normal approach to study enzyme activity is "steady-state" conditions: the concentrations of enzyme-substrate complexes remain essentially constant. The enzyme is present at a very small concentration: 0.1% or less of the substrate concentration.

Enzyme assays

3.1. Continuous

3.2. Stopped (Discontinuous)

3.1. Continuous assays

The conversion of substrate to product can be continuously observed.

Spectrophotometric (absorbance)

Most dehydrogenases used $NAD(P)^+/NAD(P)H+H^+$ redox system

$NAD(P)H$ absorbs radiation at 340 nm, $NAD(P)^+$ does not

Alcohol dehydrogenase

$Ethanol + NAD^+ \leftrightarrow acetaldehyde + NADH + (H^+)$

Fluorometric (fluorescence - molecule emits light of one wavelength after absorbing light of a different wavelength)

Calorimetric (calorimetry is the measurement of the heat released or absorbed by chemical reactions)

Chemiluminescent (chemiluminescence is the emission of light by a chemical reaction)

horseradish peroxidase + TMB substrate (ELISA)

3.1.1. Coupled assays

When the enzyme-catalyzed reaction of interest does not lead to any convenient spectroscopic signal, it is possible to couple this reaction to a second one in which such a change occurs.

Hexokinase

D-glucose + ATP → D-glucose-6-phosphate + $NADP^+$

Glucose-6-phosphate dehydrogenase

D-glucose-6-phosphate + $NADP^+$ → D-glucono-1,5-lactone-6-phosphate + NADPH + H^+

It is important that:

- a considerable excess of coupling enzyme (50-times greater) be added,
- the coupling substrate be at a concentration above K_m for the coupling enzyme, and
- the coupling enzyme be free of any detectable amount of the enzyme being assayed.

3.2.2. Stopped (discontinuous) assay

Enzyme is incubated with substrate for a fixed period of time

Before analysis:

- inhibition of the enzyme
  - rapid changes in pH
  - rapid heating (90 ℃ sufficient to denaturate most enzymes)
  - addition of trichloroacetic or perchloric acid (to final c = 3–5%) – neutralization before product measurement
  - addition of SDS to 1%
- establish that:
  - the enzyme is instantaneously inactivated
  - conditions used do not lead to any breakdown of the substrate or product being measured

Radiometric assays measure the incorporation of radioactive isotopes (^{14}C, ^{32}P, ^{35}S and ^{125}I) into substrates or its release from substrates.

Chromatographic assays measure product formation by separating the reaction mixture into its components by chromatography (HPLC, RP-HPLC)

Chemical measurement (for example: phosphate + non-precipitating acid molybdate → color formation)

How to ensure that valid kinetic data is obtained

1. It is important to use buffer components and substrates of high purity (analytical grade).

2. A control experiment should be performed in the absence of enzyme.

3. Experimental variables (temperature, pH) are controlled.

4. Enzyme is stable under the conditions used for assay.

5. The initial rate should be measured.

6. The rate of the reaction is constant over the time period used to measure the rate, and this rate is proportional to the amount of the enzyme added.

4. Binding assays

Proteins bind other molecules (ligands): proteins, nucleic acids, carbohydrates, or small molecules

Quantitative analysis:

- What is the strength of the protein-ligand interaction?
- How many binding sites for the ligand are there on the protein?
- If there are multiple ligand binding sites, are they independent or are there cooperative interactions between them?
- Is the strength of the protein-ligand interactions modified by other molecules?

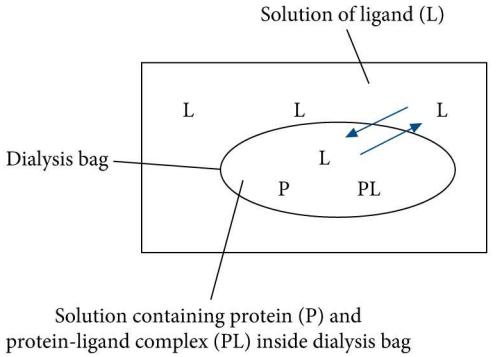
4.1. Methods involving separation of the protein-ligand complex
(on the basic of size)

4.2. Methods involving a signal change on complex formation

4.3. Competition methods

4.4. Immunochemical methods

4.1. Methods involving separation of the protein-ligand complex

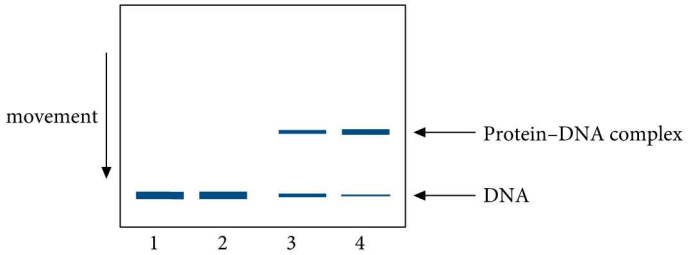


4.1.1. Equilibrium dialysis

- At equilibrium: inside $[L_t] = [L_b] + [L_f]$, outside $[L_t] = [L_f]$
  → measurement of L, PL, P concentrations
- Applications
  – to study the binding of ligands with low molecular mass

4.1.2. Electrophoretic mobility shift assay (EMSA)

- Widely used technique for studying the binding of proteins to DNA and RNA (or their fragments)
- Based on: The mobility of the nucleic acid upon electrophoresis in an agarose or polyacrylamide gel is reduced when it forms a complex with the appropriate protein.
- Can be used to monitor other types of interaction (P-P).



t: total, *b*: bound, *f*: free

4.1. Methods involving separation of the protein-ligand complex

4.1.3. Ultracentrifugation

In gravitational fields, proteins have a tendency to sediment at a rate which depends on their size and shape.

Approaches

- Measuring the concentration of small ligands (difference in L sedimentation rate (L alone and L+P) to detect its binding to protein).

- Centrifuging the mixture of P and L at a high speed for a sufficiently long time to sediment the P and PL complex and then analyze the protein-free supernatant for the free L.

- Exploring the interaction between P and a high molecular mass L (another P). Solution is centrifuged at moderate speed for several hours so that equilibrium is established between the tendency of the macromolecules to sediment to the bottom of the cell and their tendency to diffuse away from the bottom of the cell to a region of lower concentration.

4.2. Methods involving a signal change on complex formation

- Changes in fluorescence
  - (fluorescent ligands, fused proteins with GFP, YFP,..)
- Circular dichroism (CD)
  - CD depends on the differential absorption of the left- and right-circularly polarized light by a chiral chromophore. Changes in the CD spectra are directly proportional to the amount of the protein-ligand complexes formed.
- Nuclear magnetic resonance
  - The spectroscopic properties of each nucleus are sensitive to its chemical environment → can be used to monitor protein–ligand interactions and characterize them in thermodynamic terms. NMR provide great structural information about the amino acid residues in the protein that interact with the ligand.
- Isothermal titration calorimetry (ITC)
  - directly determines changes in thermodynamic parameters (ΔH, ΔG, ΔS)
- Surface plasmon resonance
  - Based on changes of the optical properties of a surface layer containing immobilized protein with increasing mass of protein bound from mobile fluid phase.

4.3. Competition methods

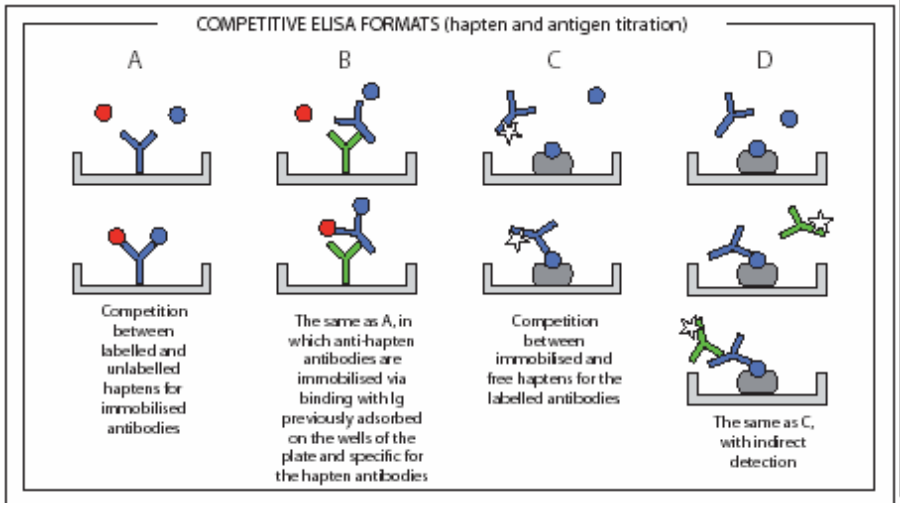
When a ligand does not show an appropriate signal change (for example, fluorescence) upon binding → competition of the ligand with a reference one of the same binding site that shows such change.

4.4. Immunochemical assays

Based on the specific binding of an antibody (Ab) to an antigen (An).

4.4.1. Enzyme-linked immunosorbent assay (ELISA)

- Ag and Ab detection and quantification
- Detection and quantification of protein-protein interactions



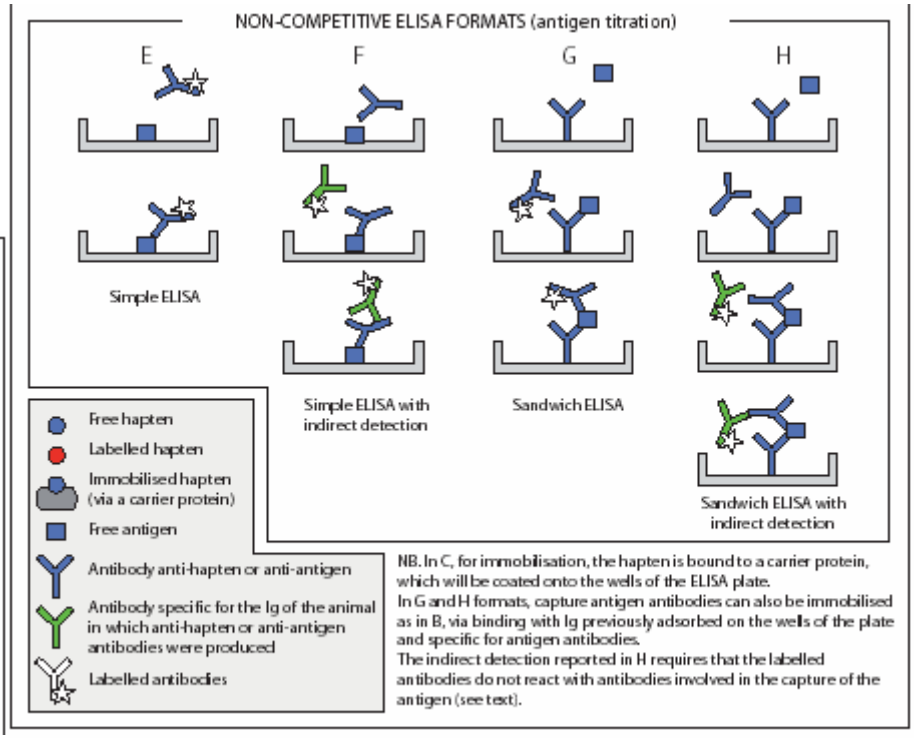
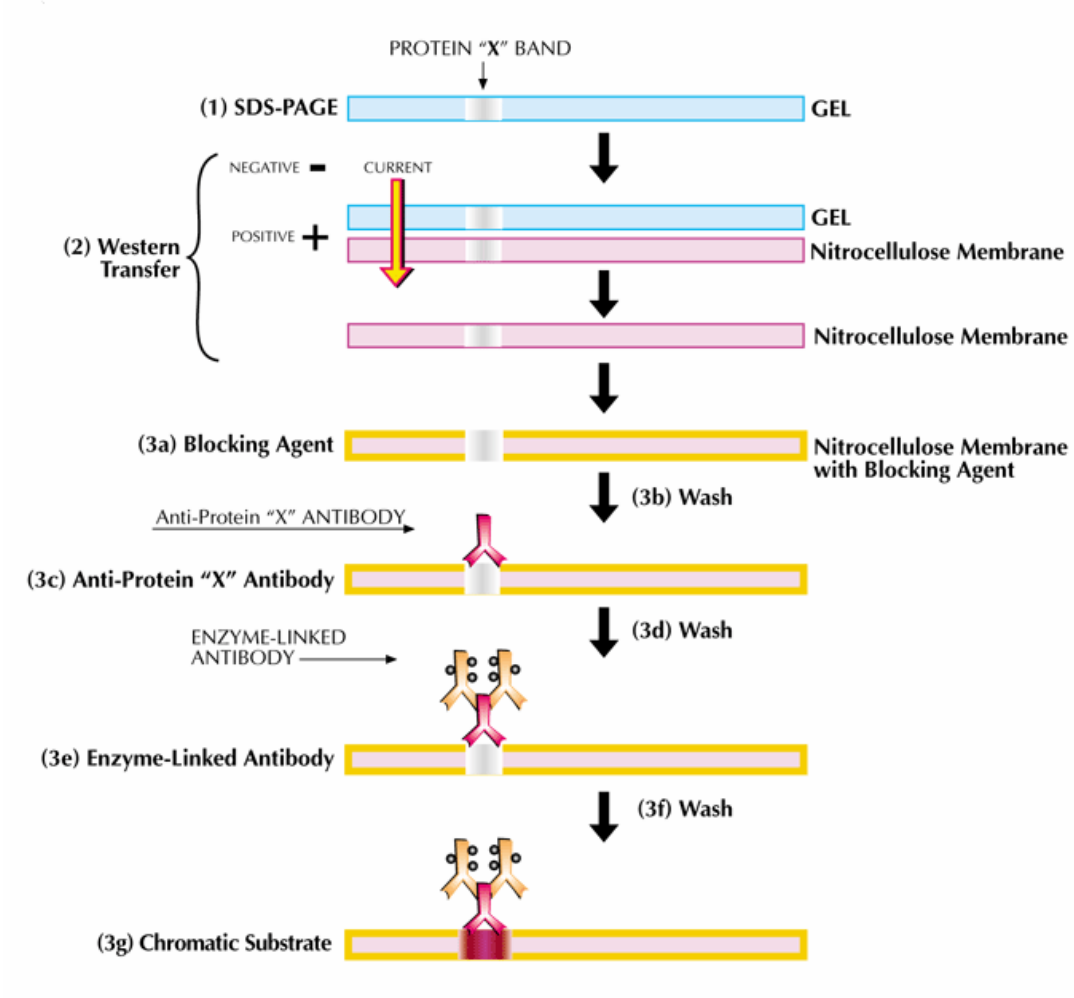


Figure 22. Different formats of the two ELISA groups. In these sketches, the first step represents the wells coated with the immobilised reactant and blocked (saturated).

Daussant & Desvaux, 2007

4.4.2. Western blot

After gel electrophoresis proteins are transferred onto a membrane, they are detected using specific antibodies.



Membrane: NC, PVDF
Blocking agent: non-fat milk, BSA
Enzyme: alkaline phosphatase horse radish peroxidase
Detection: colorimetric or chemiluminescent

Blot overlay (Far Western blot)

To study protein-protein interactions, proteins transferred to membrane are overlaid with a soluble protein that bind to one or more immobilized proteins. Overlaid protein is detected via incubation with antibodies.

Electrotransfer

1. Semi-dry

has lower buffering capacity and thus is not suitable for prolonged transfer

2. Wet tank transfer

Towbin transfer buffer, pH 8.3

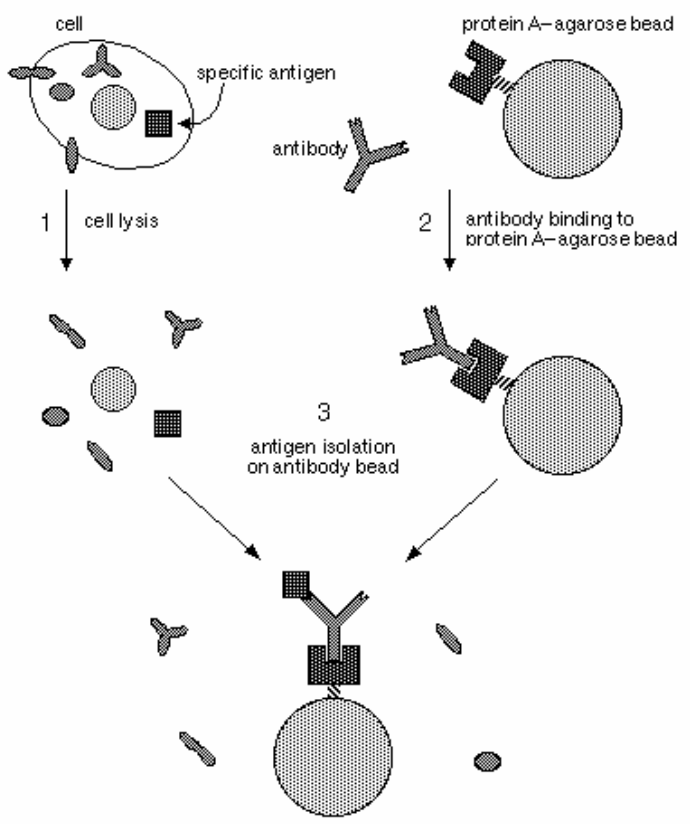
If a protein has pI equal to the buffer pH, transfer will not be promoted. Higher pH buffers such as CAPS or lower pH buffers such as acetic acid solutions can be used.

High Mw proteins or membrane proteins may be very hydrophobic and exhibit solubility problems in the absence of SDS → SDS in buffer < 0.05%.

Transfer efficiency: reversible Ponceau S red staining of membrane and/or gel staining

4.4.3. Immunoprecipitation

Isolation of antigen (protein) from a non-homogeneous solution, such as a cell lysate, using specific antibodies. Ag-Ab complex is captured by Ab-binding proteins (like protein A or G), which should only bind the antibody and whatever is associated with it. The bound Ag-Ab complex is subsequently released from the Ab-binding molecule and analyzed by SDS-PAGE and Western blot.



Applications

- to determine the presence and quantity of proteins
- to determine protein molecular mass
- to determine protein-protein interactions
- to determine specific enzymatic activity