Separation of proteins by polyacrylamide gel electrophoresis

Laboratory course – Specials methods

For master course of chemistry Department of chemistry-Analytical chemistry. spring semester

1. Theory of SDS-PAGE

Gel electrophoresis belongs to electromigration analytical methods; is a procedure for separating a mixture of molecules through a stationary material (gel) in an electrical field.

Analytes are separate by effect of DC electric field in electrolytic solution according their various mobilities. Migration characteristics of the analytical species depends on the charge, size and shape of the molecule, media conditions (p*H*) and the strength of electric field. The electrophoretical mobility (migration velocities) μ of the charged particles can be defined as ion velocity v in the electrical field with-unit intensity E:

$$\mu = \frac{v}{E} \tag{1}$$

Relation between ion charge Q and velocity v is given by another equation:

$$\mu = \frac{Q}{6\pi \cdot \eta \cdot r \cdot v} \tag{2}$$

According to equation, the bulk particles with small charge will be move slowly than non bulk particles with large charge. Mobility unit is $cm^2 \cdot s^{-1} \cdot V^{-1}$.

Gel electrophoresis (next only GE) can be classified from the different views; according to experiment setup, we resolve horizontal or vertical electrophoresis. The slab gels can be developed ether horizontally or vertically. The buffer system determines the power requirements and affects separation. The buffer system is composed of the buffer used in the gel and running buffer. There are continuous and discontinuous buffer systems. Discontinuous buffer system consists from two gel parts, stacking gel for preconcentration and resolving gel for protein separation. Gel electrophoresis resolves

into SDS PAGE (GE in dissociating or denaturing system) and Native PAGE (GE in non-dissociating or non-denaturing system) according the environment in which samples are separation.

GE is used for determination of the analyt molecular weigh by comparison of retentive factors of the protein of interest and the standard. Retentive factor is directly depended up/related to distance migrated by the protein of interest *a* and undirectly/inversely related up distance migrated by the ion front *b*. End of separation can be detected by species, which is not attached in the gel (e.g. bromophenol blue or methylene blue).

$$R_f = \frac{a}{b} \tag{3}$$

PAGE means PolyAcrylamide Gel Electrophoresis. Polyacrylamide is the matrix of choice for separating proteins. It is chemically inert, easy to handle, and transparent. Acrylamide (AA) is monomer of this gel. Acrylamide is toxic when is ingested and can be absorbed through the skin. We work with him with preventative (glove, laboratory coat). Polyacrylamide gels are formed from the copolymerization of the acrylamide monomer and a bifunctional cross linking agent to form a complex uniform web-like polymer, the porosity of which depends on the concentration of the monomers. Radical polymerization will be waged with catalyst and with free radical. Ammonium persulfate $(NH_4)_2S_2O_8$ is a strong oxidizing agent. It is a radical initiator. Tetramethylethylenediamine (*TEMED*), a catalyst for polyacrylamide gel polymerization, removes oxygen which obstructs the polymerization.

Polyacrylamide gels are characterized by %T and %C. The %T is the weight percentage of the total monomer including the crosslinker and gives an indication of the relative pore size of the gel. %T is calculated using the following:

$$\%\, T = \frac{g(AA) + g(BIS) \cdot 100\%}{ml(Vtotal)} \tag{4}$$

%C is the crosslinker:acrylamide monomer ratio of the monomer solution. %C is calculated using the following equation:

$$\%C = \frac{g(BIS) \cdot 100\%}{g(AA) + g(BIS)} \tag{5}$$

We used T = 30 % a C = 2.67 %.

Electrophoresis in denaturing conditions SDS PAGE separate proteins only according to their molecular size. In contrast to SDS PAGE, the mobilities of proteins in a Native PAGE system depend on both size and charge.

SDS is derivated from sodium dodecyl sulfate. It is a detergent that can dissolve hydrophobic molecules but also has a negative charge (sulfate) attached to it. All proteins have a large negative charge which means they will all migrate towards the positive pole when placed in an electric field. Detergent relates to the protein in amount 1.4 mg to 1 mg of protein. When dissociating conditions are to be used the protein samples are usually boiled for several minute in sodium dodecyl sulphate to ensure denaturation and treated with 2-mercaptoethanol to reduce the disulphide bonds that cross link some polypeptides.

The most popular discontinuous system employed is the SDS PAGE buffer system by Laemmli.

Non-dissociating conditions, which maintain the native structure of the protein, proteins retain their native conformation and hence their functions. Key parameters in this system are p*I* protein of interest and p*H* of the electrophoresis buffer. The p*H* of the electrophoresis buffer must be within the p*H* range over which the protein interest is stable and retains biological activity. A buffer with a p*H* greater than the protein p*I* will impart a negative charge on the protein and it will migrate toward the positive electrode (anode). A p*H* equal to the p*I* will result in no net charge in the protein and it will not migrate in an electric field.

Many dyes have been used to proteins staining. Commonly used are silver, Coomassie Brilliant Blue G-250 and R-250 and SYPRO RUBY staining. The next gel adaptation designates about choice of protein staining. CBB R-250 is used for MALDI-TOF MS. The staining by silver is better for hyphenated technique GE with laser ablation with inductively coupled plasma mass spectrometry.

1.1. Theme goals

Separation of unknown sample by polyacrylamide gel electrophoresis and identify a sample according to standards ladder.

2. Performance of SDS-PAGE

Used devices:

Mini-Protean® 3 Cell (Bio-Rad. Philadelphia. USA)

PowerPac Basic Power Supply (Bio-Rad. Philadelphia. USA)

Shaker CFL (Schüttelapparate Shakers. Burgwedel. Germany)

p*H* metrer (CyberScan p*H* 510 a Ion 510. Helago. Czech Republic)

Balance (Ohasius. Vitrum. Czech Republic)

The laboratory course is ordered to nest parts:

2.1. Used chemicals and solutions

2.2. Sample preparation

2.3. Preparation of polyacrylamide gel for separation with denaturing conditions.

2.4. Gel visualization

2.5. The question for discussion

Note. We work with acrylamide all the time only in gloves. (It means. all manipulation with AA as are weighing. preparating. staining. etc.)!!!!!!!!!!!!!!

2.1. Used chemicals and solutions

These solutions are needed for process gel electrophoresis. We check presence of all solutions at the beginning of laboratory course. Eventually we prepare the absent solution.

i) Stacking Gel Buffer 0.5 mol.l^{-1} Tris-HCl, pH 6.8:

Dissolve 6.00 g Tris into 60 ml of water. Adjust to p*H* 6.8 with 6 mol.l^{-1} of HCl and bring total volume up to 100 ml with deionized water and store at 4 °C in plastic vessel.

ii) Resolving Gel Buffer 1.5 mol.l^{-1} Tris-HCl, pH 8.8:

Dissolve 27.26 g Tris into 80 ml of deionized water. Adjust to p*H* 8.8 with 6 mol.l^{-1} of HCl and bring total volume up to 150 ml with deionized water and store at 4 °C in plastic vessel.

iii) 10xElectrode Running Buffer, pH 8.3:

Dissolve 30.30 g of Tris and 144.00 g of glycine and 10.00 g of SDS into 850 ml of dd. water. Is not requirement adjusting p*H*! Bring total volume up to 1000 ml with dd. water. One electrophoresis run needs 10times diluted the buffer to volume 500 ml. Store at 4 °C in plastic vessel.

iv) 10% APS:

Dissolve 100 mg of APS into 1 ml of dd. water. Prepare daily fresh solution!

v) Acyrylamide/Bis (30 %T. 2.67 %C):

Dissolve 87.6 g of acrylamide and 2.4 g of *N´,N´*-bis-methylenacrylamide into 200 ml of dd. water. Filter and bring total volume up to 300 ml with dd. water. Store at 4 °C in plastic vessel (max. 30 days).

vi) Sample buffer, SDS Reducing Buffer:

Give 3.55 ml of dd.water and 1.25 ml of 0.5 mol.l^{-1} Tris-HCl p*H* 6.8 and 2.50 ml of glycerine and 2.00 ml of 10% (w/v) SDS and 0.2 ml of 0.5% (w/v) bromphenol blue and bring total volume into 10 ml. Store at room temperature.

vii) 10% SDS:

Dissolve 10 g of SDS into 90 ml dd. water with gentle stirring and bring to 100 ml with dd. water.

2.2. Sample preparation

Standards of proteins: BSA (bovine serum albumin). (Sigma. Steinheim. Germany)
Myoglobin (from horse). (Sigma. Steinheim. Germany)
Cytochrom C (from horse heart). (Sigma. Steinheim. Germany)

WORK with 2-merkaptoethanol IN THE FUME HOOD!!!!

Prepared solution of the standard with concentration 0.0001 mol.l^{-1} is mix in double with 2-mercaptoethanol and sample buffer in the 500 µl microcentrifuge tube (reagents in 5.25:1 ratio, respective. Prepare the volume for five dose of reagent in one tube for protein denaturation. Suitable mark the tube and insert it into warm water bath (temperature about 95°C) for five minutes. The reducing of disulphide bonds occurs in the protein owing to bath. Inject volume 15 µl of the sample into individual wells after cooling. Calculate final concentration of protein standard into individual wells (it means amount of the separations protein).

2.3. Preparation of polyacrylamide gel for separation with denaturing conditions

***Tab. 1**. Composition of gel of various gels concentrations.*

Percentage of gel [%]	ddi H_2O [ml]	30% Degassed Acrylamide/Bis [ml]	*Gel Buffer [ml]	10% w/v SDS [ml]
4	6.1	1.3	2.5	0.1
5	5.7	1.7	2.5	0.1
6	5.4	2.0	2.5	0.1
7	5.1	2.3	2.5	0.1
8	4.7	2.7	2.5	0.1
9	4.4	3.0	2.5	0.1
10	4.1	3.3	2.5	0.1
11	3.7	3.7	2.5	0.1
12	3.4	4.0	2.5	0.1
13	3.1	4.3	2.5	0.1
14	2.7	4.7	2.5	0.1
15	2.4	5.0	2.5	0.1
16	2.1	5.3	2.5	0.1
17	1.7	5.7	2.5	0.1
18	1.4	6.0	2.5	0.1
19	1.1	6.3	2.5	0.1
20	0.7	6.7	2.5	0.1

* Stacking Gel Buffer 0.5 $mol.l^{-1}$ Tris-HCl. p*H* 6.8

* Resolving Gel Buffer 1.5 $mol.l^{-1}$ Tris-HCl. p*H* 8.8

Polyacrylamide gel is starts with pouring the freshly homogenous mixed mixture between glasses/plates, which may be fixed to Casting Stand. Place a short plate on top of a spacer plate. Insert both plates into the green Casting frame on a flat surface. Be sure that the "legs" of the casting frame are down. Clamp the Casting frame and check that the plates are level on the bottom.

Determine the appropriate gel type and composition for your experiment. At the first, start with resolving/separating gel buffer (s. table), furthermore add always the same amount of TEMED (5 µl) and 10% APS (50 µl). Combine all reagents (except TEMED) in small beaker in the order listed. When ready to pour the gel, quickly add the TEMED, mix by swirling gently, draw the solution into a 10 ml syringe and gently dispense the solution between the glass plates completely filling the space between the plates. Eject the remaining acrylamide solution back into the small beaker.

* Allow gel to polymerize (~ 60 min)

* The gel is covered by butanol layer against air access

The second, prepare stacking gel buffer (for composition of gel s. table), furthermore add always the same amount of TEMED (10 µl) and 10% APS (50 µl). Follow at preparation as before. Stacking gel size is shorter and smaller than size of separation gel. Again dispense the solution between the glass plates and completely filling the space between the plates. Insert the well forming comb into the opening between the glass plates. No air must be there! Eject the remaining acrylamide solution back into the small beaker. Polymerization of this solution indicates the completeness of polymerization of the gel between the plates.

* Allow gel to polymerize (~ 45 min)
* Once the gel has polymerized, the comb can be gently removed. The polymerized gel between the short plate and spacer plate forms the "gel cassette".
* The plates take down from Stand.

Remove the gel cassette sandwich from the casting frame. Place gel cassette sandwich into electrode assembly with the short plate facing inward. Slide gel cassette sandwiches and electrode assembly into the clamping frame. Press down on the electrode assembly while closing the two cam levers of the clamping frame. This step is important to minimize potential leakage during the electrophoresis experiment. Lower the inner chamber into the electrophoresis mini tank.

The sample loading guide located the sample wells. Insert Hamilton syringe into the slots of the guide and fill the corresponding wells by 10-15 µl of denaturated sample. Load samples slowly to allow them to settle evenly on the bottom of the well. Wash the syringe 4times at least before reusing!

Then fill the inner chamber into mini tank with cca. 125 ml of running buffer until the levels reaches halfway the tops of the taller and shorter glass plates of the gel cassettes. Add cca. 200 ml of running buffer to the mini tank (lower buffer chamber). Place the lid on tank. Make sure to align the color coded banana plugs and jacks. Add electrode to electrical current source and set constant voltage and focusing time. **Isotachophoresis running at 100 V for 10 minutes, separation at 145 V for 30 minutes.**

When the electrophoresis is complete, turn off the power supply, disassemble the apparatus and clean all relevant equipment. Remove the gels from cassette by gently separating the two plates of the gel cassette. The green, wedgeshaped, plastic releaser may be used to help pry the glass plates apart. The end of separation is marked by cutting into gel. The gel in Petri dish is fixed according used visualization.

2.4. Gel visualization

Gel Coomassie Brilliant Blue R-250staining protocol:

1) Fixing solution: 45% methanol and 10% acetic acid
(450 ml methanol and 100 ml acetic acid in 1000 ml)

2) Staining solution: 45% methanol and 10% acetic acid and 0.1% CBB
(450 ml methanol and 100 ml acetic acid and 1 g CBB in 1000 ml)

3) Destaining solution: 5% methanol and 7.5% acetic acid
(50 ml methanol and 75 ml acetic acid in 1000 ml)

Sink the gel into fixing solution for 30 minutes. Then pour off the solution above gel and sink the gel into staining solution. Give the Petri dish with gel into fridge at 4 °C for 3 hours. Then cast the staining solution and wash gels with water. The gel in Petri dish is destained in destaining solution at shaker. Shake with gel gently, be careful so that it broken. Change the destaining solution as often as will be requirement. It means, that protein bands will be only blue and gel background only transparent. Store gel in Petri dish in destaining solution at 4°C.

Silver staining protocol:

1) Fixing solution: methanol:acetic acid:water in rate in the 45:2:45 ratio

2) 0.02% solution $Na_2S_2O_3$ (0.1 g of $Na_2S_2O_3$ dissolve and bring total volume in 500 ml)

3) 0.1% solution Ag (0.5 g of $AgNO_3$ dissolve and bring total volume in 500 ml)

4) Developing solution (12.5 g of Na_2CO_3 and 150 µl of 37% formaldehyde and bring total volume into 500 ml)

5) Stop solution (1% acetic acid)

Sink the gel into fixing solution for 20-30 minutes at 4 °C. Then wash the gel with water several times. Give the gel into 0.02% solution of sodium thiosulphate for 2 minutes. Replace in 0.1% solution of silver nitrate and incubate for 30-40 minutes at 4 °C. Wash the gel with water several times. Sink the gel developing solution and shake with dish in the hands. This step is very important for staining. The gel becomes slightly yellow. Change the solution as often as will be requirement. Stop reaction with 1% acetic acid. Store the gel in Petri dish in this acid at 4°C.

Pour off silver solutions to selected/marked waste!

2.5. The questions for discussion

1) Which substances have been presented at polymerization and what is their function?
2) Compare method of gel and capillary electrophoresis, their advantages and disadvantages. What is mutual characteristic?
3) Staining with CBB R-250 and silver, explain which of these is more suitable for MALDI-TOF and/or LA-ICP mass spectrometry?

Protocol evaluation

In protocol introduce your name and date; make one protocol for one group.Add experimantal results:

* Calculate R_f of separated proteins from scan gel.
* Identify protein/s of unknown sample and make statistical evaluation with standards.
* Calculate final concentration of protein standard into individual wells, it means amount of the separations protein.

Answer to the questions for disscussion and discusses results.

Recommended literature

J. D. Hayes, P. K. Stockman, *Br. Medj.*, 1898, **299**: 843-846.

U. K. Laemmli, *Nature,* 1970, **227**: 680-685.

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