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High-Resolution Two-Dimensional Polyacrylamide Gel Electrophoresis: A Tool for Identification of Polymorphic and Modified Linker Histone Components

Andrzej Kowalski and Jan Pałyga
Department of Biochemistry and Genetics, Institute of Biology, Jan Kochanowski University, Poland

1. Introduction

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) is a powerful technique used for separation, detection and quantification of complex protein mixtures expressed within cellular systems. Whereas a single-dimension electrophoretic separation allows to detect individual proteins in cell extracts mainly based on a single physicochemical parameter only and may be therefore inadequate for effective resolution of protein components, two-dimensional analysis is fully capable of simultaneous separation of majority protein constituents employing at least two independent parameters for their separation. Depending on the nature of protein complexes to be resolved, 2D-PAGE may comprise isoelectric focusing (IEF) or acetic acid-urea polyacrylamide gel electrophoresis (AU-PAGE) in the first dimension followed by sodium dodecyl suflate polyacrylamide gel electrophoresis (SDS-PAGE) in the second one. Therefore, this technique combines protein fractionation according to isoelectric point in the IFE or size and net charge in the AU-PAGE with the separation in conformity with their molecular weights in the SDS-PAGE gels. A combination of the IEF in the first dimension with the SDS-PAGE in the second dimension, originally introduced by O'Farrell (1975) for total cellular protein separation in *Escherichia coli*, is currently widely used in the majority of proteome analyses (Görg et al., 2004; Weiss & Görg, 2009). The fusion of AU-PAGE with the SDS-PAGE is especially handy for the identification of charged proteins of low to medium size in heterogeneous protein preparations containing a limited number of components such as cellular acid-soluble proteins including histones (Goldknopf & Busch, 1975). The one-dimension electrophoretic techniques separate the bulk of protein into distinct units independently of their conformation and keep them from forming the aggregates. At highly acidic pH of AU-PAGE system, around pH 3.5, the positively charged proteins of similar size can be resolved from each other according to their charges. Addition of urea which is an efficient agent for breaking non-covalent protein interactions improve the protein solubility and facilitates electrophoretic migration. At an alkaline pH of the SDS-PAGE system, routinely above pH

8.0, the negative charge proportional to the mass of separated protein is imparted by the SDS-dependent denaturation. Owing to binding to SDS molecules, the protein chains acquire a uniform negative charge allowing their separation by mass alone. However, determination of accurate molecular weight by SDS-PAGE may be inadequate for proteins rich in proline residues, such as fibrous proteins, or possessing a high number of positively (histones) or negatively charged groups (phosphoproteins) that tend to alter the rate of their in-gel migration (Simpson, 2003). A combination of AU-PAGE system (Panyim & Chalkley, 1969) that separates the proteins based on differences in their size and effective charge with a discontinuous SDS-PAGE system (Laemmli, 1970) capable of resolving proteins according to their apparent molecular masses is a suitable approach for a comprehensive examination of genetically heterogeneous and chemically modified protein components (Mizzen, 2004). A coupling of these two electrophoretic techniques, using a slightly modified method (Pałyga, 1991a), has been successfully adopted for detection and characterization of linker histone polymorphic variants that differ in size and charge. This electrophoretic system is relatively straightforward and reproducible. Beside qualitative and quantitative applications (Kowalski et al., 2011a, 2011b) for protein detection and subsequent abundance estimation, the 2D-PAGE-like method may also be used on a preparative scale to enable gathering and purification of appropriate amount of the resolved protein for subsequent structural analyses including chemical cleavage and enzymatic digestion (Górnicka-Michalska et al., 1998; Pałyga & Neelin, 1998; Pałyga et al., 2000; Kowalski et al., 2011a, 2011b) as well as amino acid sequencing (Górnicka-Michalska et al., 2006) and mass spectrometry analysis (Kowalski et al., 2009).

Although a resolving power of this 2D-PAGE technique might be limited by inability of separating proteins that are less basic and/or of larger size, these restrictions do not apply to the linker histones. During our screening of the linker histone preparations in the 2D-PAGE we often revealed protein spots which had not been separated well in one-dimensional AU-PAGE and SDS-PAGE because they co-migrated with the adjoining protein bands (Kowalski et al., 2008, Kowalski et al., 2010). Usually, the 2D-PAGE patterns of linker histones represented a diagonal arrangement of non-overlapping protein spots. The use of this technique was also helpful for detection of allelic and/or posttranslationally modified forms of linker histone subtypes undetectable in the first dimension acetic acid-urea polyacrylamide gel and visible as single or double protein spots when resolved in the second dimension in the polyacrylamide gel containing SDS (Kowalski et al., 2009).

Despite novel approaches adopted for efficient fractionation of various protein mixtures that display specific advantages and disadvantages, the 2D-PAGE which links AU-PAGE in the first dimension with the SDS-PAGE in the second dimension may still remain an essential technique for characterization the basic protein preparations like histones allowing for precise identification of their individual components.

2. Materials and equipment

2.1 Apparatus

While commercially available standard equipment may be suitable for a simultaneous separation of a large number of preparations in a long gel, we adopted a modified

electrophoretic system of Kerckaert (1978) that suited best for our purposes of efficient population screening. In this type of apparatus (Fig. 1), the gels are polymerized between quadratic (24 cm x 24 cm) 4 mm-thick glass plates separated by teflon spacers of variable thickness depending on the type of the electrophoretic run. The thinner spacers (0.7 mm) are used for the first dimension separation while thicker ones (0.9 mm) are employed for the second dimension. Before gel polymerization, the glass plates separated by the spacers are placed inside a 50 cm-long propylene plastic bag and then stabilized by broad rubber bands and metal clamps. In order to cast the gel, the assembly is vertically positioned on the bench using a laboratory stand.

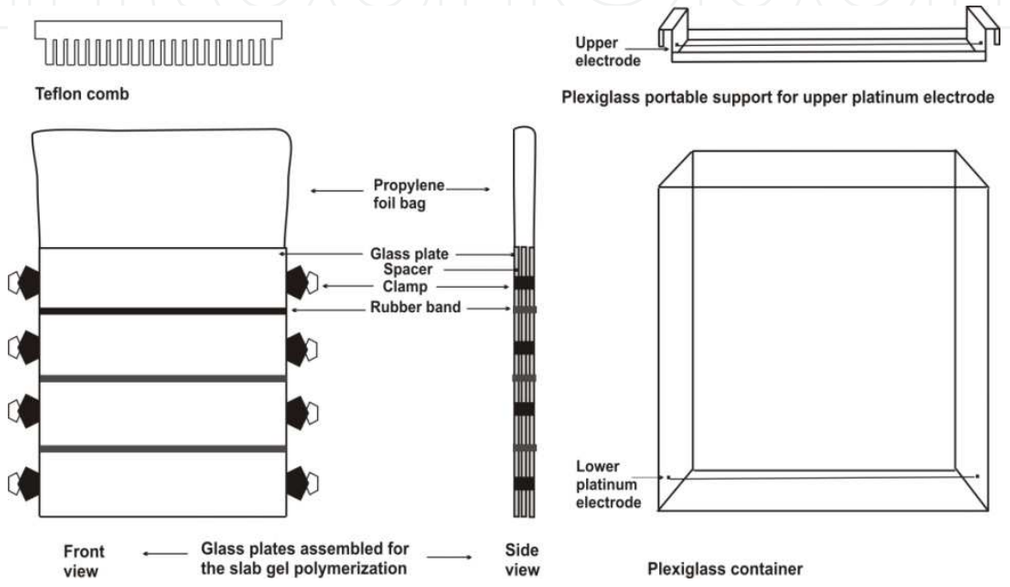


Fig. 1. A schematic representation of the assembly for one and two-dimensional -PAGE system. A details concerning dimensions of individual elements, such as glass plates, teflon combs and plexiglass container, and the order of assembly are given in the text (see section 2.1).

After polymerization of the gel, the clamps are removed and a bottom part of the plastic bag is cut off to enable a flow of the current. Then, the assembly containing the polymerized gel(s) is inserted into a plexiglass vessel containing platinum electrode stretched at its base. The container is filled with a running buffer to three quarters of its volume. The upper part of the plastic bag (about 30 cm) protruding above the glass plates is then carefully spread inside the vessel so that it is lying above the buffer surface, and fixed to its walls by plastic clips to form an upper buffer reservoir. A plexiglass portable support with a platinum electrode inside is then inserted from the top and the upper reservoir is filled with the buffer. Such a design enables separation of up to twenty samples in one slab gel when protein preparations are to be resolved in the first dimension and from seven to eight gel stripes with the proteins previously electrophoresed in the first dimension to resolve them further in the second dimension. In this electrophoretic system, the effective length of protein separation is at least 20 cm. A size of the plexiglass vessel (30 cm x 30 cm x 12 cm) allows a simultaneous run of up to four slab gels. However, in order to avoid overheating that produce fuzzy patterns of the resolved proteins we usually electrophoresed only two

gels in a single run. The spacers (0.5 x 24 cm) cut from a teflon stripe are 0.7-mm thick for the first dimension AU-PAGE and 0.9-mm thick for the second dimension SDS-PAGE. The combs used for the first dimension AU-PAGE, made from a 0.7-mm thick teflon sheet, have 5-mm wide and 12-mm deep teeth separated by 3 mm gaps. While about 4 – 6-μL aliquots of dissolved protein preparation is usually loaded into the wells cast in the comb gel, the volume of the wells allows loading up to 20 μL of the preparation when the protein concentration in the sample is low.

2.2 Chemicals

The majority of reagents used to prepare buffers and solutions for electrophoresis, i.e. acrylamide (4 x cryst., analytical grade), *N,N'*-methylene-bisacrylamide (2 x cryst., analytical grade), *N,N,N',N'*-tetramethylethylenediamine (TEMED), 2-amino-2-hydroxymethyl-propane-1,3-diol (Tris base), sodium dodecyl sulfate (SDS) (2 x cryst., premium grade) and urea (analytical grade), were usually purchased from SERVA Electrophoresis GmbH (Heidelberg, Germany) but alternatively we have also applied the chemicals from other suppliers (Merck Chemicals, Sigma-Aldrich, etc.) with the same effect. The ammonium persulfate (APS), glacial acetic acid and glycine were obtained from POCH (Gliwice, Poland). Routinely, Millipore purified water for making buffers and solutions was employed.

2.3 Solutions

2.3.1 Acrylamide gel stock solutions

Apart from 2% (v/v) TEMED solution for SDS-PAGE made by pipetting the appropriate volume of TEMED and adjusting to a final concentration with water, the remaining solutions: acrylamide–*N,N'*-methylene-bisacrylamide (40% acrylamide–1.34% *N,N'*-methylene-bisacrylamide for AU-PAGE and 30% acrylamide–0.8% *N,N'*-methylene-bisacrylamide for SDS-PAGE), Tris buffer (1.5M Tris-HCl, pH 8.8 for running gel and 0.5 M Tris-HCl, pH 6.8 for stacking gel of SDS-PAGE) and 1% SDS were prepared by weighing the appropriate amount of the reagent and mixing with a small volume of water. After complete dissolution of the substance water was added to the desired final volume. The acrylamide and bis-acrylamide were dissolved by a mechanical or magnetic stirring with water and the resulting solution was filtered. The stock solutions for gel polymerization were used without extra purification step (for recipe, see Table 1).

2.3.2 Staining solutions

For slab gel staining and destaining with Coomassie Brilliant Blue (CBB) R-250 we consecutively used a staining solution (0.05% CBB R-250, 25% 2-propanol, 10% acetic acid), staining/destaining solution (0.0035% CBB R-250, 10% 2-propanol, 10% acetic acid) and destaining solution (10% acetic acid). The staining and staining/destaining solutions were prepared by a mechanical (or magnetic) stirring until CBB dye was completely dissolved (for recipe, see Table 2).

Colloidal Coomassie Brilliant Blue (CBB) G-250 staining solution was made as follows: 0.1% CBB G-250 solution in water was added to previously dissolved 10% ammonium sulfate in

water and then the mixture was supplemented with 3% *ortho*-phosphoric acid and 20% ethanol (for recipe, see Table 2).

For protein staining with silver, 0.1% silver nitrate, 12% trichloroacetic acid, 3% potassium carbonate containing 0.05% formalin and 1% acetic acid were used as staining, fixing, developing and stopping solutions, respectively. All reagents were prepared freshly just before use (for recipe, see Table 2).

Component	Amount	Concentration	Final volume
Acrylamide-bisacrylamide for AU-PAGE[a)]	200g–6.7 g	40%–1.34%	500 mL
Acrylamide-bisacrylamide for SDS-PAGE [a)]	150g–4 g	30%–0.8%	500 mL
Tris-HCl, pH 8.8[b)]	90.85 g	1.5 M	500 mL
Tris-HCl, pH 6.8 [c)]	15.14 g	0.5 M	250 mL
SDS	1 g	1%	100 mL
TEMED	1 mL	2%	50 mL

Table 1. Recipes and notes for stock solutions. The provided final volumes of the solutions are sufficient for polymerization of about ten slab gels. Notes: [a)]Potential neurotoxins, working with gloves and a mask protecting the face is recommended. [b) and c)]pH should be adjusted at room temperature by adding 6M HCl, about 30 mL to b) and 20 mL to c) after dissolving the reagent. The pH of the buffer should be monitored using a pH-meter.

Coomassie Brilliant Blue (CBB) R-250[a)]		
Staining solution	Staining/Destaining solution	Destaining solution
0.05% CBB R-250	0.0035% CBB R-250	10% Acetic acid
25% 2-Propanol	10% 2-Propanol	
10% Acetic acid	10% Acetic acid	
Coomassie Brilliant Blue (CBB) G-250[b)]		
Staining solution	Destaining solution	
0.1% CBB G-250	Water	
10% Ammonium sulfate		
3% *ortho*-Phosphoric acid		
20% Ethanol		
Silver staining[c)]		
Staining solution	0.1% Silver nitrate	
Fixing solution	12% Trichloroacetic acid	
Developing solution	3% Potassium carbonate – 0.05% formalin	
Stopping solution	1% Acetic acid	

Table 2. Recipes and notes for staining solutions. Note [a)] For complete dissolution of CBB R-250, the use of magnetic stirrer is recommended. [b)] The aqueous solution of CBB G-250 should be mixed with a completely dissolved solution of ammonium sulfate in water. [c)]All reagents should be prepared fresh before use.

2.4 Running buffers

A 0.9 M acetic acid solution was used as an electrode buffer for one-dimension AU-PAGE. For a single electrophoretic run in one-dimension SDS-PAGE or second dimension of the 2D-PAGE, a running buffer (0.192 M glycine, 0.025 M Tris-base and 0.1% SDS) was prepared by dissolving buffer components in about 4-5 L of water and then diluted to the final volume of seven liters (for detailed recipe, see Table 3).

The gel stripes with stained protein bands were cut out from one-dimension acetic acid-urea gel with a razor blade and then adapted for second dimension SDS-PAGE in an equilibration buffer containing 100 mM Tris-base, 10% glycerol, 2.1% SDS and 2% 2-mercaptoethanol. The aqueous solution of Tris-base was adjusted to pH 6.8 with appropriate volume of 6 M HCl solution [see note b) in the Table 3] before adding the rest of the compounds and supplemented with water up to 100 ml (for recipe, see Table 3).

The protein preparations were solubilized in a buffer containing 8 M urea, 0.9 M acetic acid and 10% 2-mercaptoethanol made by initial dissolving of urea with water and subsequent addition of the remaining ingredients (for recipe, see Table 3).

Component	Amount	Final concentration
AU-PAGE electrode solution		
Glacial acetic acid	360.5 mL/7 L	0.9 M
SDS-PAGE running buffer[a)]		
Glycine	100.8 g	0.192 M
Tris-base	21 g	0.025 M
SDS	7 g	1%
Equilibration buffer[b)]		
Tris-base	1.5 g	100 mM
Glycerol	10 mL	10%
SDS	2.1 g	2.1%
2-mercaptoethanol	2 mL	2%
Sample buffer		
Urea	24 g	8 M
Glacial acetic acid	2.6 mL	0.9 M
2-Mercaptoethanol	10 mL	10%

Table 3. Recipes and notes for running, equilibration and sample buffers. The running buffer is made by dissolving the ingredients in water to the final volume of seven liters for a single electrophoresis run. The components of equilibration buffer were dissolved in the final volume of 100 mL which is enough to adapt about fifty gel stripes intended to resolve in the second dimension in six slab gels. The final volume of sample buffer is 50 ml. Note [a)] The intensive foaming of SDS delays and impedes dissolution of the remaining components. Mix SDS separately with the 3 – 4 liters of water, then add glycine and Tris-base separately dissolved in the 1 liter of water, and supplement to the final volume of 7 L. Note [b)] The pH value of the buffer should be 6.8. Initially, dissolve Tris-base in 40 ml of water and add 2.125 ml 6M HCl to obtain the right pH value. Then, add glycerol, SDS, 2-mercaptoethanol and water up to 100 ml. Store at 4°C.

3. Preparation and running of 2D-page

Both one- and two-dimension electrophoresis is carried out in a 20 x 20 cm running gel polymerized between 24 x 24 cm glass plates. A comb gel is then cast on top of the running gel by pouring 8% polyacrylamide solution up to the height of 3 cm. The volumes of all solutions needed for casting two gel slabs are given in Table 4. The final polymerizing mixture (except ammonium persulfate) is carefully degassed under water vacuum pump, usually from several minutes to half an hour, until all air bubbles are removed.

3.1 Sample preparation

Perchloric acid-soluble protein samples isolated from saponin-purified avian erythrocyte nuclei (Neelin et al., 1995), which contain mainly linker histones, are made ready for electerophoresis by dissolving 1-mg portions of protein preparations in 200 μL sample buffer (Table 3) and incubation at 37°C for 2 – 4h or alternatively at room temperature for about 8h. For a better visibility of sample aliquots during loading, a bit of pyronin G or other suitable dye is added to the protein samples. The aliquots are loaded into the gel wells using Hamilton microsyringe or narrow pipette tips, typically in the volume of 4 – 6 μl.

3.2 First dimension AU-PAGE

The running gel is prepared by adding 41.25 ml 40% acrylamide–1.34% *N,N'*-bisacrylamide stock solution, 52.8 g urea, 5.71 ml glacial acetic acid and 0.55 ml TEMED to a 150-ml graduated and stoppered cylinder. After dissolving the urea the solution is supplemented with water to the final volume of 110 ml water, transferred to a conical flask and carefully degassed under water vacuum pump. Then, 1.34 ml of 10% APS is added, carefully mixed and poured along the inner edge of pre-assembled mould of two (a single gel) or three glass plates (a double gel) separated with teflon spacers, and with teflon comb(s) already inserted. To obtain a flat gel surface, approximately 0.5 ml of isobutyl alcohol is carefully overlaid along the top of the gel. Usually, the gel polymerizes for about half an hour at room temperature but a longer polymerization time (at least 1 h) is recommended.

Then, a low-porosity comb gel is made by combining 9 mL 40% acrylamide–1.34% *N,N'*-bisacrylamide, 11.5 g urea, 1.24 mL glacial acetic acid, 0.12 mL TEMED and water to the final volume of 24 mL. After dissolving, the comb gel solution is degassed and then combined with 0.3 mL 10% APS followed by a gentle mixing. Isobutanol and unpolimerized gel remnants remaining at the top of running gel are removed, the gel surface is briefly washed with water and a layer of comb gel is poured until the teeth of the inserted comb are well immersed. When this gel is ready, after at least half an hour at room temperature, the teflon combs are removed and the surfaces of formed wells are rinsed again with water. The gels are preelectrophoresed overnight in 0.9 M acetic acid at 50 V constant voltage per a single slab gel. The course of preelectrophoresis can be monitored by loading an aliquot of sample buffer containing 0.05% bromocresol green into at least one well. Since the basic protein, like histones, are positively charged at acidic pH, they will move directly from anode (+) to cathode (–). Therefore, the (+) and (–) connecting leads of a power supply should be wired to the upper and lower buffer

reservoir, respectively. After loading the protein samples, the gel is electrophoresed at 150 V constant voltage per single slab for about 48 h until the main protein band of cytochrome C loaded at the rightmost lane is running out of the gel. Cytochrome C serves here as a marker of the rate migration if the linker histones are to be separated over a long distance. To obtain a better separation of histone H1 bands, the electrophoresis run may be further prolonged allowing cytochrome C to exit completely out of the gel. Under these conditions the main and fastest linker histone of avian erythrocytes, histone H5, is usually running out. For resolving total histones, the current should be switched off when a tracking dye reaches the bottom edge of the slab gel to avoid losses of the fastest migrating polypeptides (histone H4).

First dimension AU-PAGE	
Running gel	Comb gel
40% Acrylamide–1.34% *N,N'*-bisacrylamide - 41.25 mL	40% Acrylamide–1.34% *N,N'*-bisacrylamide - 9 mL
Urea - 52.8 g	Urea – 11.5 g
Glacial acetic acid – 5.71 mL	Glacial acetic acid – 1.24 mL
TEMED (conc.) - 0.55 mL	TEMED (conc.) – 0.12 mL
10% APS – 1.34 mL	10% APS – 0.3 mL
H_2O – 62.15 mL	H_2O – 13.34 mL
Final volume – 110 mL	Final volume – 24 ml
Second dimension SDS-PAGE	
Running gel	Stacking gel
30% Acrylamide-0.8% *N,N'*-bisacrylamid - 58.5 mL	30% Acrylamide-0.8% *N,N'*-bisacrylamide – 4 mL
1.5 M Tris-HCl, pH 8.8 – 32.5 mL	0.5 M Tris-HCl, pH 6.8 – 5 mL
1% SDS – 13 mL	1% SDS – 2 mL
2% TEMED – 3.25 mL	2% TEMED – 1 mL
10% APS – 0.455 mL	10% APS – 0.06 mL
H_2O – 22.75 ml	H_2O – 8 mL
Final volume – 130.455 mL	Final volume – 20.06 mL

Table 4. Recipes for casting first and second dimension gels. The final volume is given for two gel slabs and needs to be adjusted if other electrophoretic systems are used.

3.3 Second dimension SDS-PAGE

As the first dimension gel electrophoresis and subsequent staining and destaining of resolved proteins were conducted at the acidic conditions, the gel fragments with protein bands to be separated in the second dimension must be earlier equilibrated at neutral environment, pH 6.8, corresponding to the pH value of the SDS stacking gel. For this purpose, the gel stripes containing protein bands resolved in the first dimension AU-PAGE are excised from the gel with a sharp scalpel and transferred to tubes filled with the equilibration buffer. The completely immersed gel pieces should be adapted for minimum 15 min, then the buffer is discarded and the gel pieces are equilibrated in a fresh portion of the buffer for further 15 min. It is important to leave even edges in the cut gel stripes.

Uneven edges that do not adhere strictly to top of the flat stacking gel may cause a disturbed movement of proteins entering the stacking gel and produce wavy, blurred or even illegible protein patterns that hinder interpretation of the images.

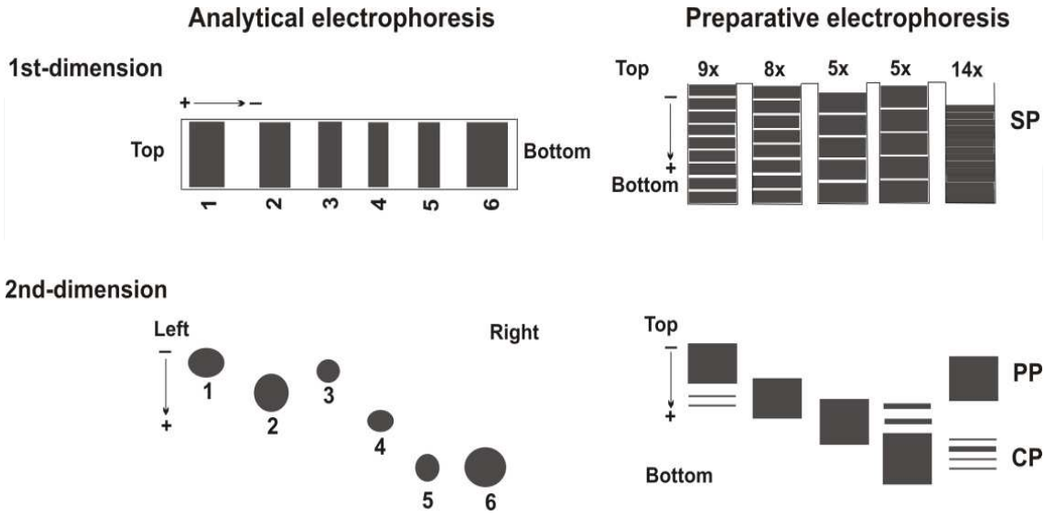


Fig. 2. A scheme presenting the way of placing the one-dimension gel pieces onto the second-dimensional gel. The vertical gel stripe(s) containing resolved protein bands, designated from 1 to 6 according to their increasing electrophoretic migration in the first dimension, cut out of the acetic acid-urea gel should be oriented horizontally from left to right (analytical approach) or accommodated in stacks containing a varying number, for example 9, 8, 5, 5, 14, of the gel stripes depending on the band size of the same protein (SP) to obtain finally a concentrated band of pure protein (PP) freed from contamination by other proteins (CP) (preparative approach). For better visualization of the gel stripe alignment in preparative electrophoresis, the gel pieces have been schematically presented as separate entities in four leftmost wells but they should strictly adhere to each other and to the gel surface as depicted in the fifth well on the right (for details see subsection 3.3).

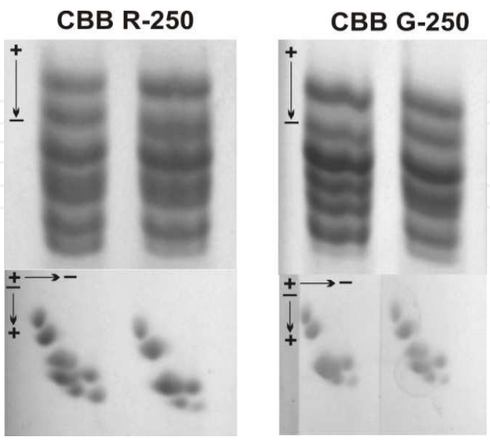


Fig. 3. A comparison of the effectiveness of quail erythrocyte H1 histone staining with CBB R-250 and CBB G-250. Both histone H1 bands separated in the first dimension AU-PAGE (upper panel) and protein spots resolved in the second dimension SDS-PAGE (lower panel) exhibit similar relative intensities after staining with either CBB R-250 or CBB G-250.

The polymerizing solution for casting the running gel is directly made of 58.5 mL 30% acrylamide–0.8% *N,N'*-bisacrylamide, 32.5 mL 1.5 M Tris-HCl, pH 8.8, 13 mL 1% SDS, 3.25 mL 2% TEMED and 22.75 mL H_2O in a conical flask. After deaeration under water suction pump, 0.455 mL 10% ammonium persulfate is added and after careful mixing the whole solution is poured to the pre-assembled mould of two glass plates separated by the spacers (0.9 mm) thicker than those used in the first dimension (0.7 mm). Isobutyl alcohol, 0.5 mL per one slab gel, is applied on top of the polymerizing gel solution. The gel is ready for running in about one hour but longer polymerizing time is recommended. The stacking gel solution consists of 4 mL 30% acrylamide–0.8% *N,N'*-bisacrylamide, 5 mL 0.5 M Tris-HCl, pH 6.8, 2 mL 1% SDS, 1 mL 2% TEMED and 8 mL H_2O. The solution is degassed prior to adding 0.06 ml 10% ammonium persulfate solution. The stacking gel is left to polymerize on top of the running gel to form a flat surface, this time without combs, that is needed to arrange equilibrated gel stripes. The direction of placing of the gel pieces on the stacking gel is as follows. The protein bands resolved in the first dimension gel from top to bottom should be placed from left to right in the second dimensional gel (Fig. 2). The gel pieces can be placed using narrow metal spatulas at the 2 – 3 mm intervals and need to adhere firmly to the surface of the stacking gel. A lack of close contacts between gel surfaces may partially restrict or fully prevent entering the protein from the stripes into the stacking gel. After arranging the stripes, the upper reservoir is carefully filled with the running buffer. During this step, the gel pieces can slide against each other, so it is important to check their position before starting the electrophoresis. To avoid accidental movement of the gel stripes they can be overlaid with a fresh portion of stacking gel and left to polymerize for additional half an hour.

For preparative electrophoresis, the stacking gel is polymerized with combs containing 7-mm wide and 30-mm deep teeth. This allows for positioning a different number, depending on the size of the band, of stacked gel pieces containing the same protein band. As a result, the protein is not only being concentrated but also separated from impurities coming from closely migrating neighboring bands in the first dimension gel (Fig. 2). For a maximum resolution, the electrophoresis is run for about 22 hours at 30 mA constant current per single slab gel. It should be pointed out that SDS is coating the proteins with a negative charge so that they run from cathode (–) to anode (+). Therefore, the electrical leads between power supply and electrophoresis system should be attached as follow: the lead (–) to the upper and the lead (+) to the lower reservoir, respectively.

3.4 Protein detection

Following electrophoresis, the glass-gel assembly is carefully opened while laying flat on the bench and the gel is carefully transferred to a plastic or glass container in which protein staining and destaining is conducted.

Despite the notion (Neuhoff et al., 1988) that the use of colloidal staining with CBB G-250 with a working detection range of 5 - 500 ng of protein improve protein visualization compared to the conventional staining with CBB R-250 with a working detection range of 100 - 1000 ng of protein, we did not observe significant differences between both staining methods (Fig. 3). As presented in Fig. 3, similar intensities of stained linker histone bands with no additional components were observed using staining with both dyes. So, both

staining techniques can be employed, although making a working solution and time spent on staining are shorter when CBB G-250 technique is applied. Staining the gels with the CBB G-250 for about two hours leads to sharp protein bands on a clear gel background, the effect which can be achieved by destaining with water for approximately four hours. Staining with CBB R-250 requires an exchange of the 0.05% staining solution first with staining/destaining solution containing less CBB R-250 and isopropanol for one hour and, after that, practically overnight destaining of the gel in 10% acetic acid to obtain well visible protein bands on a transparent gel background. In both procedures, the gel should be completely immersed in the staining solution and agitated from time to time to obtain a uniformly stained gel surface without traces of precipitated dye which can limit visibility of the protein bands.

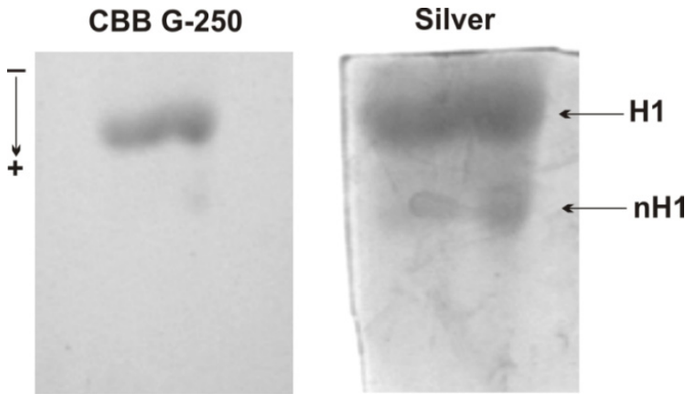


Fig. 4. Silver staining of quail histone H1.a band that was earlier visualized with Coomassie Brilliant Blue R-250. Apart from the broader band of histone H1.a, an accompanying new protein component (nH1) is now apparent.

In order to increase the sensitivity of protein detection in the gels already stained with CBB dye solutions and simultaneous detection of low-abundance protein components, the subsequent gel staining with silver is recommended. For this purpose we modified the procedure described by Blum et al. (1986). While the original procedure requires acetic acid/ethanol and sodium carbonate/formaldehyde mixtures to fix proteins and develop band color in the gels, respectively, we apply trichloroacetic acid as a fixative solution and potassium carbonate/formalin as a developer (Table 2). Before staining, the excised fragment of the gel containing proteins stained with CBB is fixed in 12% trichloroacetic acid solution (20 – 30 min). Afterwards, the gel is washed with 10% ethanol (2 x 5 min) and water (3 x 5 min). The protein is stained with 0.1% silver nitrate for 30 min, and then the gel stripe is repeatedly rinsed with water. Now, the gel stripe is immersed in the solution of 3% potassium carbonate containing 0.05% formalin for 10 min and thereafter the reaction is stopped by washing with 1% acetic acid (5 min.). Finally, the gel stripe is rinsed with water (for about 5 min) until brown-colored protein bands appear on a transparent yellowish background. Since silver staining is very sensitive, all operations should be performed with gloves to prevent dirty background that limits visibility of the stained protein profiles. The use of silver staining successfully enhances detection of protein bands in the polyacrylamide gel. As was shown in Fig. 4, a linker histone protein band first visualized with CBB appeared as a broad band following silver staining. Although the background of gel stained

with the silver is not fully clear, an additional protein band undetectable in the CBB stained gel can be seen.

3.5 Gel documentation and processing

Currently, the electrophoretic patterns of resolved proteins are usually recorded in a digital format by commercially available gel documentation systems. Captured protein patterns are then processed by computer software allowing both for direct evaluation of individual protein band and/or spot migration rates and their intensities, as well as band-to-band and/or spot-to-spot comparisons between separate gels. In our analyses that usually require estimation of differences in the range of electrophoretic mobilities and relative band densities between separated proteins, we use the Doc-Print II gel documentation system (Vilber Lourmat) for saving the gel images and ImageJ 1.42q software (www. rsbweb.nih.gov/ij) for their further processing. This allows for a precise identification of the electrophoretic mobility and estimation of the expression level of individual components in the protein samples and facilitates detection any disparities.

As was shown in Fig. 5, a densitometric tracing of protein band profiles resolved in the first dimension AU-PAGE enables estimation their relative abundance in the selected peak areas (Table 5). The intensity of the corresponding protein spots separated in the second dimension SDS-PAGE (Fig. 5) can also be evaluated by measuring a mean grey value within a selected spot or by integrated density, the sum of pixel values in the selected spot (Table 5). Thus, these measurements allow to detect differences among distinct protein components.

4. Application of 2D-PAGE

Although 2D-PAGE, just like any other separation technique, possesses a limited resolving power, it can be successfully applied to the identification of polymorphic and modified protein variants. Our 2D-PAGE procedure is especially useful for separation a bulk of avian linker histones into several non-allelic subtypes differing in a number and quantity between species (Fig. 6), which may contain extra non-allelic variants (Fig. 5), allelic isoforms of the polymorphic variants and modified forms (Fig. 7). As highly basic proteins do not differ enough in both net charge and molecular mass to be fully separated by one-dimensional AU-PAGE (Pałyga, 1991b) or SDS-PAGE (Kowalski et al, 1998), the effective resolution is often achieved by using 2D-PAGE in which non-allelic members of linker histone differing in both parameters are visible.

As shown in Fig. 5, the full complement of linker histone isolated from grey partridge erythrocytes contains seven non-allelic subtypes. Six of them are visible as prominent protein bands resolved in the first dimension acid-urea polyacrylamide gel due to differences in their net charges. The seventh minor histone H1.a' was detected only in the second dimension polyacrylamide gel due to its co-migration with the histone H1.a in the first dimension polyacrylamide gel. Thus, the histone H1.a' may share a similar net charge with histone H1.a but differs in the molecular weight. On the other hand, histone H1.b' migrating as a well separated band in the first dimension polyacrylamide gel differs from the histone H1.b in the net charge but exhibits a similar molecular mass so that these proteins form a partially overlapping spot in the second dimension polyacrylamide gel

(Fig. 5). A further screening of histone H1.a′ spots in grey partridge population using 2D-PAGE revealed a polymorphism reflected by the presence of allelic variant H1.a′1 and H1.a′2 existing either in the form of homozygous phenotypes a′1 and a′2 or heterozygous phenotype a′1a′2 (Kowalski et al., 2008).

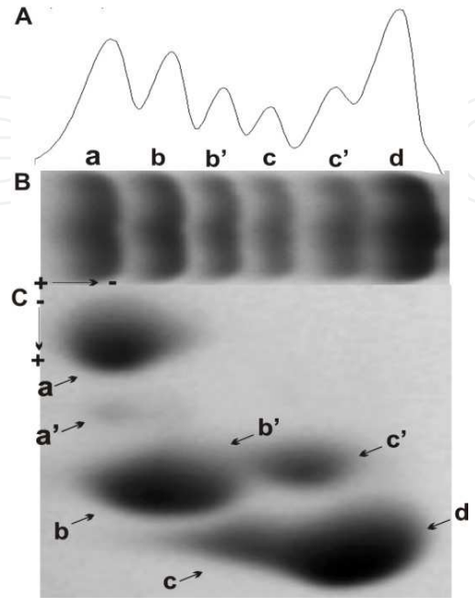


Fig. 5. The complement of grey partridge histone H1 resolved in the 2D-PAGE. (A) Densitometric tracing of respective histone H1 bands (H1.a, H1.b, H1.b′, H1.c, H1.c′ and H1.d) resolved (B) in the first dimension AU-PAGE. (C) A faintly stained extra spot of minor subtype H1.a′ is discernible in the second-dimension SDS-PAGE pattern of total histone H1.

Histone H1 subtype	First dimension AU-PAGE Area Percentage		Second dimension SDS-PAGE Mean grey value	Integrated density
H1.a	19253.045	21.577	123.799	12.657
H1.a′[a)]				
H1.b	15655.125	17.544	145.776	7.833
H1.b′	9402.397	10.537	149.968	8.220
H1.c	6841.983	7.668	161.475	8.178
H1.c′	10990.761	12.317	158.243	8.676
H1.d	27087.752	30.357	111.882	12.510

Table 5. Abundance of linker histone non-allelic subtypes in grey partridge erythrocytes. The amount of protein in linker histone bands was assessed by measuring peak area in the densitometric tracing of one-dimension AU-PAGE gel profiles. The determined peak areas were used to calculate the percentage of each protein component in the total protein loaded onto the gel. The intensities of selected protein spots in the second-dimension SDS-PAGE gel profiles were presented both as mean grey level values or integrated spot densities representing the sum of pixels values. [a)] The abundance of faintly stained spot of histone H1.a′ visible only in the second dimension SDS-PAGE was not measured due to its low intensity compared with the gel background (see Fig. 5).

Therefore, a prerequisite for pursuing histone allelic variants, some of which may differ in the electrophoretic migration in two-dimensional polyacrylamide gel, is to establish first a full pattern of effectively resolved non-alellic linker histone variants. Two-dimensional electrophoretic patterns of linker histone polymorphic subtypes differing with net charge or molecular weights are schematically depicted in Fig. 8 A and B. Besides presented examples of histone H1.z possessing two allelic variants in Pekin duck (Fig. 8 C) (Pałyga et al., 1993) and three allelic variants in Muscovy duck (Fig. 8 D) (Kowalski et al., 2004) which differ either in molecular weight or molecular weight and net charge, respectively, the polymorphisms of duck, Guinea fowl and grey partridge histone H1.b (Pałyga et al., 2000; Kowalski et al., 2011a, 2011b), pheasant and Guinea fowl histone H1.c (Kowalski et al., 2010; Kowalski et al., 2011a) have been detected using two-dimensional electrophoresis. A high resolution 2D-PAGE is also necessary to confirm the presence of allelic variants when some of them are faint or missing because of close migration in a direct vicinity of adjoining protein band in the first dimension polyacrylamide gel (Kowalski et al., 2010). Beside identification of new allelic components, the 2D-PAGE protein patterns may facilitate assessment of relative protein spot abundance (see subsection 3.5, Table 5) that allows a statistical evaluation of the protein variability between the samples (Kowalski et al., 2011).

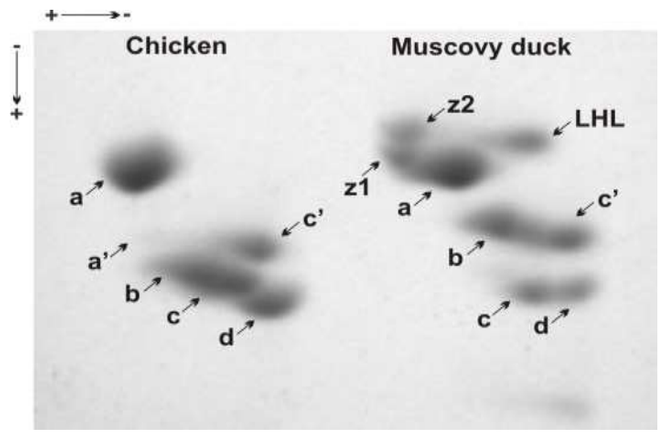


Fig. 6. Species-specific patterns of avian erythrocyte histone H1 subtypes revealed in the 2D-PAGE. Both avian species possess histone H1 subtypes H1.a, H1.b, H1.c, H1.c' and H1.d, while subtype H1.a' was seen within the array of chicken histone H1 and subtype H1.z, here represented by a heterozygous phenotype z1z2, was revealed in the set of duck H1 subtypes only. A linker histone-like (LHL) band, likely representing a posttranslationally modified form of histone H1, was found in Muscovy duck only.

Two-dimensional PAGE can also be used as a preparatory step for protein identification in gel pieces by mass spectrometry (Fig. 9) (Górnicka-Michalska et al., 2006; Kowalski et al., 2009) or for gathering a greater amount of concentrated protein to protease cleavage (Górnicka-Michalska et al., 2006; Kowalski et al., 2011) (Fig. 10) and microsequencing (Górnicka-Michalska et al., 2006). In this type of 2D-PAGE, the protein band(s) previously resolved in AU-PAGE or SDS-PAGE are vertically laid in the wells of stacking gel (see subsection 3.3 and Fig. 2) in order to obtain a homogeneous protein band freed from other proteins contaminants, if any (Fig. 10).

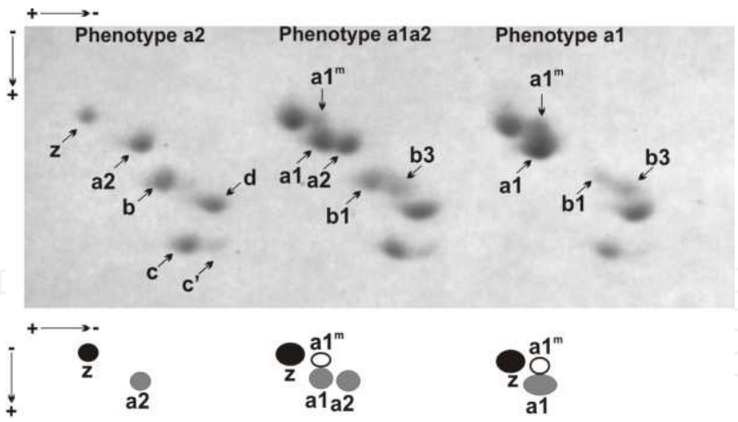


Fig. 7. 2D-PAGE patterns of allelic variants (H1.a1 and H1.a2) and presumable modified forms of duck histone H1.a subtype. The allelic variant H1.a1 seems to be partially modified judging from an aberrant migration forming a spot $a1^m$. On the ideogram, the elliptic spots represent in-gel location of histone H1.a allelic variants (grey ovals) in respect to adjacent histone H1.z (black ovals), and a presumable modified form, $a1^m$, of the histone H1.a1 (white ovals). The modified spot a^m was never seen in ducks with homozygous phenotype a2.

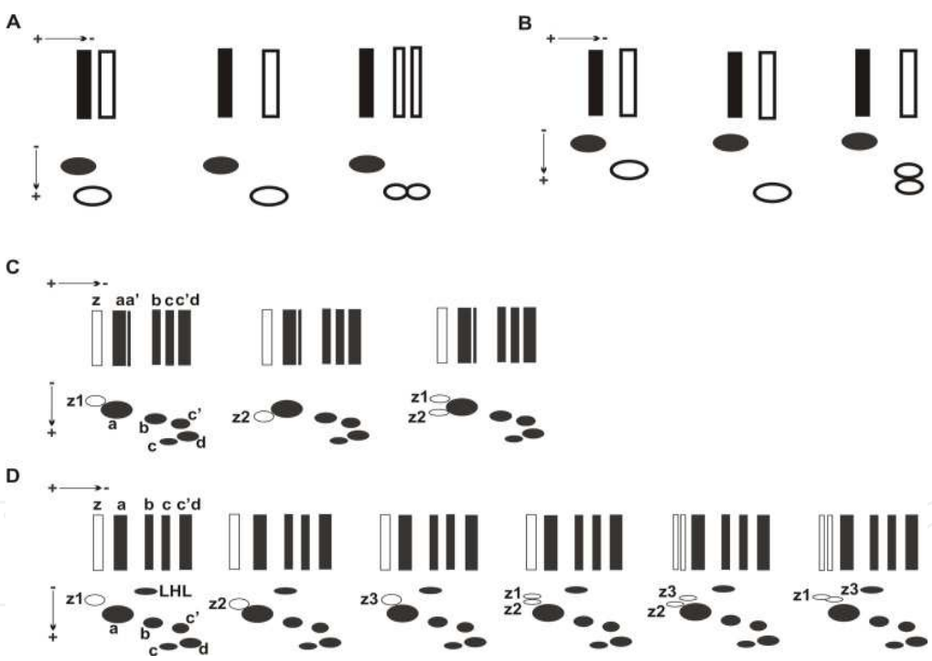
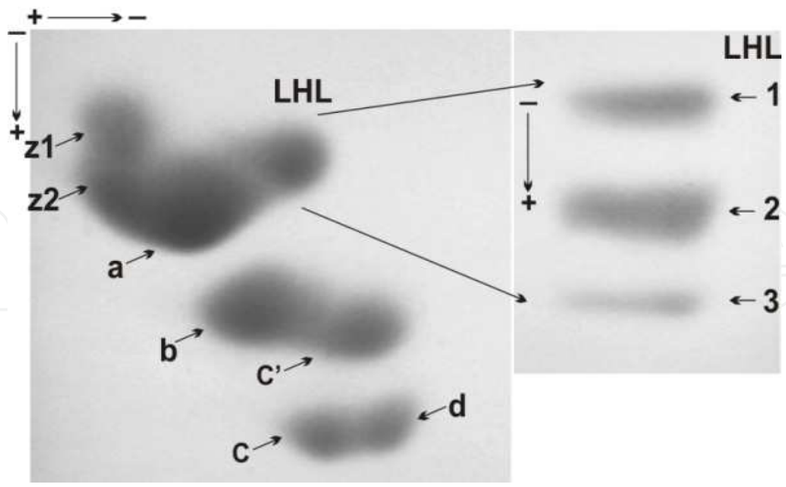


Fig. 8. A scheme presenting a general arrangement of histone H1 allelic variants in 2D-PAGE due to differences in their net charge (A) or molecular weight (B), and the ideograms depicting bi-allelic (C) and triple-allelic (D) system of histone H1.z variation identified in Pekin (Pałyga et al., 1993) and Muscovy duck (Kowalski et al., 2004) erythrocytes (D), respectively. The bands and spots of monomorphic histone H1 subtypes are marked with filled shapes while bands and spots of polymorphic subtypes are indicated with open shapes. Single spots of polymorphic histone H1 subtypes represent homozygotes while double spots correspond to heterozygotes. All H1.z phenotypes in both duck populations were identified using the 2D-PAGE.

A



B

Mr (expt)	Mr (calc)	Delta	Score	Peptide
852.405	852.409	-0.003	56	GVGASGSYR
1107.608	1107.604	0.005	40	VGQHADLQIK
1871.978	1871.956	0.022	60	KPASHPSYSEMIVAAIR

C

Cairina moschata histone H5 (accession number P06513)

MTDSPIPAPAPAAKPKRAKAPR**KPASHPSYSEMIVAAIR**AEKSRGGSSRQSIQKYVKSHY
K**VGQHADLQIK**LSIRRLLAAGVLKQTK**GVGASGSYR**LAKGDKAKKSPAGRKKKKKAARRS
TSPRKAARPRKARSPAKKPKAAARKARKKSRASPKKAKKPKTVKAKSLKTSKVKKAKRSK
PRAKSGARKSPKKK

Fig. 9. Electrophoretic detection of Linker Histone-Like (LHL) proteins within the complement of Muscovy duck histone H1 subtypes (A), a Mascot search report of LHL3 trypsin-peptides (B) and mass spectrometry assignment of LHL3 peptides to the linker histone H5 (B, C). A single LHL spot cut out from the 2D-PAGE was next separated as three distinctly migrated protein bands in one-dimensional SDS polyacrylamide gel (A). The LHL3 trypsin-peptides were first analyzed by LC LTQ FT MS/MS (Liquid Chromatography Linear Quadrupole Ion Trap Fourier Transform Mass Spectrometry) and then matched at the highest Mowse score of 220 by Mascot search engine (www.matrixscience.com) to the sequence of Muscovy duck (*Cairina moschata*) histone H5 (C) derived from NCBI (National Center for Biotechnology Information) database. The LHL3 peptides, bold characters in (C), were assigned with the individual ion scores 60, 40 and 56 to the histone H5 (B). The individual ion score higher than the threshold score of 54 means identity or extensive homology. The identities of LHL1 and LHL2 were not pursued further but we believe that they may likely represent histone H5 with a greater number of modifying groups attached.

Even though SDS-PAGE theoretically separates proteins according to their molecular sizes, many proteins can also migrate depending on their hydrophobicity (Shirai et al., 2008). Therefore, the anomalous, either faster or slower, electrophoretic mobility may reflect the presence of some modified protein forms. They can be visible as small protein spots accompanying abundant non-modified parental proteins, just like LHL (linker histone-like) adducts of linker histone subtype probably with ADP-ribose (Fig. 6 and 9) (Kowalski et al., 2009) or as protein band(s) moving faster than main protein component, like phosphorylated forms of murine histone H1 (Lennox et al., 1982). It seems likely that additional protein component a1^{m} accompanying allelic variant H1.a1 (Fig. 7) can represent a modified form of the histone H1.a1.

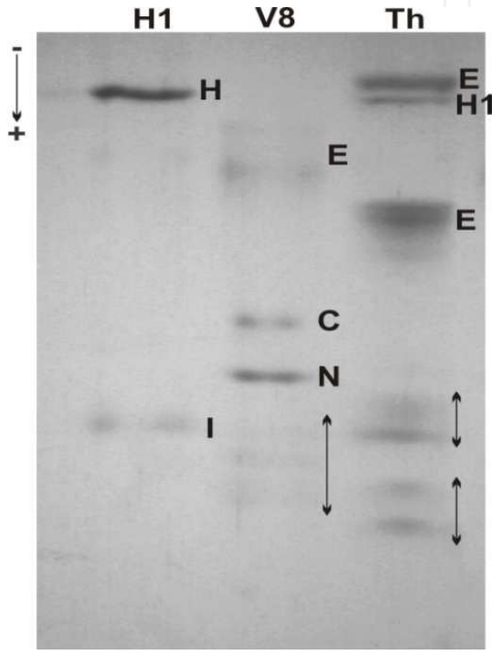


Fig. 10. The use of 2D-PAGE for protein purification and subsequent protease cleavage. A homogeneous histone H1 band (H) freed from impurities (I) was subjected to cleavage with protease V8 (lane V8) and thrombin (lane Th) into specific C-terminal (C) and N-terminal (N) fragments and less defined (double-sided arrows) minor peptides; E – protein bands of the enzyme, H1 – undigested histone H1.

5. Conclusion

One-dimensional electrophoretic techniques AU-PAGE and SDS-PAGE capable of resolving cell proteins mainly according to net charge and molecular weights, respectively, once coupled together provide a sensitive high resolution 2D-PAGE system for comprehensive separation of complex protein mixtures. A relative ease of implementation and operation of 2D-PAGE system for effective resolution of a large number of samples at a time makes it suitable for population screening of polymorphic proteins whose allelic isoforms can be identified based on differences in their net charges in the acid-urea gel and molecular weights in SDS gel. This approach enables separation not only abundant protein components but also minor forms that usually are not well-

resolved or discerned using one-dimensional techniques. Additional advantage of 2D-PAGE as an effective separation method is a possibility of estimation the relative densities of separated protein spots to assess rough levels of their expression. Apart from analytical approaches, 2D-PAGE can also be used on a preparative scale to obtain a larger amount of pure protein of interest for structural analysis. In this perspective, the 2D-PAGE may assist the other techniques of protein examination, such as mass spectrometry, microsequencing and immunodetection. The presented examples of the application of 2D-PAGE method confirm its effectiveness as a tool that can help clarify the genetic phenomena and epigenetic processes related to linker histone variants. As such events require a participation of multiple histone and non-histone proteins, the use of highly efficient 2D-PAGE system may significantly enhance their separation, detection and quantification.

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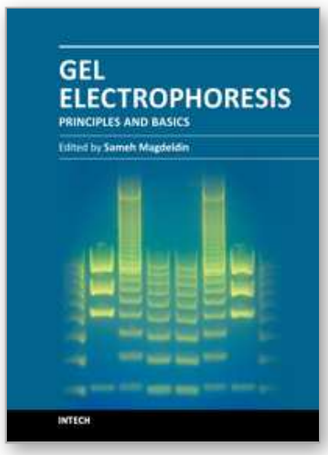

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Most will agree that gel electrophoresis is one of the basic pillars of molecular biology. This coined terminology covers a myriad of gel-based separation approaches that rely mainly on fractionating biomolecules under electrophoretic current based mainly on the molecular weight. In this book, the authors try to present simplified fundamentals of gel-based separation together with exemplarily applications of this versatile technique. We try to keep the contents of the book crisp and comprehensive, and hope that it will receive overwhelming interest and deliver benefits and valuable information to the readers.

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