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Common artifacts and mistakes made in electrophoresis

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Summary

Proteases that act at room temperature upon proteins in the sample buffer prior to heating, cleavage of the Asp-Pro bond upon prolonged heating of proteins at high temperatures, contamination of sample or sample buffer with keratin, leaching of chemicals from disposable plastic ware, contamination of urea with ammonium cyanate are some of subtle artifacts that can have significant deleterious effects on carefully planned and executed experiments. In addition, researchers are culpable of committing mistakes with respect to calculating the cross-linking factor of a gel, polymerization temperature and time for a polyacrylamide gel, inducing aggregates in samples for electrophoresis, titrating the running buffer in electrophoresis, proper sample preparation, amount of protein to be loaded on a gel, sample buffer-to-protein ratios, incompletely removing phosphate buffered saline from cells prior to cell lysis and over-focusing of IPG strip in two dimensional gel electrophoresis. Taking proper heed to all these factors can greatly help generate perfect experimental results.

Keywords

Artifacts; Common mistakes in electrophoresis; Aggregates; Keratin; Asp-Pro bond; Proteases; Ammonium cyanate; Cross-linking factor

1. Introduction

Polyacrylamide gel electrophoresis (PAGE) is an invaluable technique for investigating the protein repertoire of a cell in health and disease. Westermeier (1) and Burgess (2) have recently reported regarding frequently made mistakes in electrophoresis and important but little known artifacts in protein biochemistry. Erroneous protocols abound, in spite of widespread information available via text books, instrument manuals, and online tutorials (1). Sometimes subtle and obscure artifacts can significantly affect the outcomes of carefully performed experiments (2). This chapter details important artifacts and commonly made mistakes that can derail an otherwise perfectly executed experiment.

2. Artifacts in gel electrophoresis

2.1. Sample preparation for SDS PAGE

Burgess (2) describes three ways in which one may find contaminants in a purified or nearly purified protein in sodium dodecyl sulfate (SDS) PAGE.

2.1.1. Proteases—Protein samples are normally added to sample buffer, containing SDS, β -mercaptoethanol or dithiothreitol, sucrose or glycerol and heated at 95-100 °C for 5 min. The heating is carried out to enable better denaturation and reduction of the proteases and thus bring about its inactivation (3). Gradually, it was recognized that the heating had to be carried out as soon as the samples were diluted in the sample buffer. This is because SDS, even though it easily unfolds most proteins, does not unfold some proteases. Consequently, the proteases will have time to digest the proteins of interest in the sample buffer if the heating step is not carried out immediately. One can design an experiment to find out if proteases are causing multiple bands to appear in SDS-PAGE analysis of a purified protein or a nearly pure protein. One way would be add the protein of interest to two portions of the sample buffer. Mix well and heat one portion immediately. Leave the other at room temperature for 2-4 h and then heat. Analyze both samples on SDS PAGE and check for degradation of the protein in the sample not heated immediately. As little as 1 pg protease in a protein sample has been shown to bring about major degradation, if the sample is added to the lysis buffer and not heated immediately (2).

2.1.2. Cleavage of Asp-Pro bond at 100°C—The aspartic acid-proline bond is the most susceptible bond for cleavage by heat or acidic conditions (4). The bond is cleaved by heating at high temperatures for too long. Heating at 75°C for 5 min instead of 95-100°C for 5 min has been found to avoid D-P bond cleavage, as well as completely inactivating proteases (2). However, it has to be also noted that several proteins have been found to be stable at 100°C for several hours (5).

2.1.3. Contamination of SDS lysis buffer or sample with keratin.—Keratin runs as a heterogeneous cluster of contaminating bands around 55-65 kDa on reducing SDS gels (with β -mercaptoethanol) and near the top of the gel on non-reducing SDS gels (without β -mercaptoethanol). Keratin occurs in skin, dander, etc. Commonly the lysis buffer itself gets contaminated with skin contact or by a flake of dandruff. This is normally a minor contaminant and can be visualized mainly in silver stained gels and occasionally on Coomassie Blue stained gels. Contamination of lysis buffer with keratin can be ruled out by running sample buffer alone without the addition of proteins to a lane in the gel. The buffer should be remade if keratin bands are observed in this lane. Aliquoting lysis buffer and storing them at -80°C is practiced by many laboratories. An aliquot can be thawed and used within a day or two. Keratin has been observed occasionally in western blots as a result of contamination of antigen used to prepare a polyclonal antibody and reacting with keratin transferred from SDS-gel to membrane (2).

2.2. Leaching of chemicals from plastic ware

Certain chemicals leach out from common laboratory plastic ware (disposable) into standard aqueous buffers. In some cases this can have significant effects on the results of experiments. Chemicals like oleamide are employed as lubricating agents in the molding process. Other chemicals, such as certain cationic biocides, are used to help prevent bacterial colonization of the plastic surface. Most of this material can be removed by washing the plastic ware in water or even better with methanol or DMSO (2,6).

2.3. Contamination of urea with cyanate

Urea is used widely as a denaturing agent for proteins. However, urea solutions contain significant amounts of ammonium cyanate. Ammonium cyanate is in chemical equilibrium with urea [$(\text{H}_2\text{N})_2\text{C}=\text{O}$ in equilibrium with $\text{NH}_4^+ + \text{NCO}^-$]. Isocyanic acid ($\text{H}-\text{N}=\text{C}=\text{O}$) has been shown to react with lysine's ϵ -amino group and the amino terminus as well as with arginine and cysteine to a lesser extent to form a carbamylated protein (7). Carbamylation can interfere with enzyme function in some cases, alter charge and block certain protease cleavage reactions. In addition, as measured by mass spectrometry, it can add 43 Da per carbamylation event to the mass of the protein. To remove these contaminant ions (to diminish or prevent carbamylation) one can treat a urea solution with a mixed bed resin (Bio-Rad AG 501-X8). However, since this is a chemical equilibrium, the ammonium cyanate level builds up again to levels in the 0.5–3 mM range in an 8 M urea solution, with the potential to reach up to 20 mM (8). Chemical scavengers like ethylenediamine, glycylglycine, or glycylamide (in the 5–25 mM range) has been shown to reduce cyanate to less than 0.1 mM in 8 M urea, Tris-HCl (pH 8) (8). Burgess (2) suggests that the best general practice, if urea is to be used, is to replace some of the NaCl in the buffer with some ammonium salt (like 25–50 mM ammonium chloride) to push the equilibrium back by the common ion effect toward less cyanate. Lower temperature and acidic conditions have been shown to slow the reaction of isocyanic acid with amino groups in proteins. This effect can be also minimized by restricting the time of protein exposure to the urea solution to the shortest time possible (2).

3. Commonly made mistakes in electrophoresis

3.1. Sample preparation

Some commonly made mistakes (9) during sample preparation include employing an incorrect protein-to sample buffer ratio, failure to remove insoluble material, and overloading and under loading of protein. In order to prevent inadequate sample buffer-to-protein ratios, overloading, and under loading of samples, the protein concentration of the sample should be determined using a standard protein assay (*see Chapter 3*).

Distorted, poorly resolved bands in the overloaded lane and distorted protein bands in adjacent lanes result from loading too much protein. On the contrary, under loading leads to lack of detection of minor protein bands, and even makes major bands too faint for photographic reproduction. One should load purified protein in the 0.5–4.0 μg range (depending on well size and gel thickness) and from 40–60 μg for crude samples if a Coomassie Blue stain is to be used for gel staining. Less protein per sample is required when

silver staining method is employed, since it is about 100-fold more sensitive than Coomassie staining (9). Enough sample buffer should be used in order to maintain an excess of SDS. Most proteins bind SDS in a constant mass ratio of 1.4 µg SDS per 1.0 µg protein. However, Hames (10) recommends a ratio of 3:1.

Certain proteins like histones and membrane proteins may need addition of 6–8 M urea or a nonionic detergent such as Triton X-100 since they may not completely dissolve by heating in SDS sample buffer alone (11,12). The insoluble material should be removed by a 2-min centrifugation (at 17,000 x g) following heat treatment in SDS lysis buffer. Streaking within the gel will happen if precipitated insoluble material is not removed. If the supernatant is not loaded immediately, it can be stored at 4°C overnight or frozen at –20°C for longer periods. However, the stored samples need to be warmed briefly at 37°C to redissolve the SDS and centrifuge once again to remove insoluble material before loading (9).

Pre-treatment methods such as lyophilization, spin concentration, dialysis against concentrated polyethylene glycol (PEG), and excess solvent absorption by exposing dialysis bag with sample to dry PEG, Aquacide or Sephadex® (gel filtration media) can be employed to concentrate samples that are too dilute for analysis. Prior to addition of SDS sample buffer, samples concentrated by the above said methods may be dialyzed against 50 mM Tris-HCl, pH 6.8 to get rid of low molecular weight contaminants. Trichloroacetic acid or acetone can be used to precipitate and concentrate proteins from dilute samples and acidic samples as well as get rid of contaminants such as potassium, guanidine hydrochloride, or ionic detergents from samples (1,13).

Extremely viscous samples such as crude cell extracts owe their viscosity to the high concentration of unsheared nucleic acids. Treatment of samples with Benzonase® Nuclease (recombinant endonuclease) prior to addition of sample buffer can help eliminate the viscosity. This endonuclease lacks proteolytic activity and completely degrades all forms of DNA and RNA. Vigorous vortexing of the heated sample or physical shearing of the nucleic acids through sonication can also help reduce viscosity (9).

3.2. Miscalculating cross-linking factor of a polyacrylamide gel

Two factors control the pore size of a polyacrylamide gel, (a) the total concentration of acrylamide T and (b) the degree of cross-linking C:

$$T = \frac{(a + b) \times 100}{V} [\%], \quad C = \frac{b \times 100}{a + b} [\%]$$

a = mass of acrylamide in g

b = mass of methylenebisacrylamide in g

V = volume in mL

However, sometimes one mistakenly assumes that the given total concentration of acrylamide T is the percentage of acrylamide per volume and C (the cross-linking factor) is the percentage of methylenebisacrylamide per volume. This will lead to too much amounts

of cross-linker in the gel. This will result in gels that are opaque, brittle and highly hydrophobic. The correct method is to prepare the solutions as per the equation noted above. Alternatively, it would be better to use commercially available acrylamide/methylenebisacrylamide stock solutions that are ready-to-use (1).

3.3. Temperature and time of polymerization for a polyacrylamide gel

It takes about 30 minutes to one hour for the polymerization of acrylamide and methylenebisacrylamide to occur. The matrix formation is unfinished at this time point, however. Polymerization continues, in a silent manner for several hours for the complete matrix formation. In general, complete polymerization is brought about efficiently, only when it is carried out at room temperature (1). However, Haeberle (14) has described a high temperature SDS-PAGE (running gels at 70°C) that can be run in as little as 5 min for a mini gel. An added advantage of this method is the greatly accelerated rate of polyacrylamide cross-linking. According to this paper, the gel can be transferred to the 70°C buffer as soon as it becomes rigid enough to be removed from the casting stand and by the time the samples are loaded the cross-linking is almost complete (14).

Polyacrylamide gels are polymerized in the refrigerator or cold room in some laboratories or the gels are used one or a few hours following the initiation of polymerization. Using incompletely polymerized gels can lead to disturbances during the protein separation, especially on a native gel. In addition, high background noise will be a problem in downstream mass spectrometry analysis as a consequence of the incompletely polymerized acrylamide mono- and oligomers. The correct way is to allow the gel to polymerize overnight at room temperature. The gel can be stored in a refrigerator, if necessary, afterwards (1).

3.4. Protein aggregates in SDS samples

After boiling, the sample is often directly loaded on the SDS gel after it has cooled down. Consequently, the reductant often becomes partly oxidized, a part of the cysteines remain unprotected and results in back-folding and the creation of inter-polypeptide aggregates. Back-folding leads to blurred zones and sometimes the formation of double bands. While some aggregates precipitate in the sample well (too big to enter the gel), some induce artifactual zones in the high molecular weight area. Two or three horizontal lines across the entire gel in the molecular weight range between 40 and 60 kDa are formed by an excess of reductant. The right procedure is to allow the sample cool to approximately 60°C (after boiling) and then add iodoacetamide [10 µL of a 20 % (w/v) aqueous iodoacetamide solution to 100 µL sample] and incubate the sample for 30 minutes at room temperature. Sharper bands are obtained with this procedure and artifacts (double bands, additional high molecular weight bands, precipitate in the sample well and lines across the gel) are abolished (1).

3.5. Titrating running buffer in SDS PAGE

The discontinuous buffer system based on Laemmli's method (15) consists of the stacking and resolving gel buffers that are composed of Tris and chloride buffers with defined pH values (with or without SDS). The running buffer has SDS, Tris and glycine. The

discontinuity between the chloride (acting as the leading ion) and the glycine (acting as the trailing ion) permits a controlled slow protein entry into the polyacrylamide gel and the stacking effect, resulting in very sharp and well resolved zones.

However, some protocols call for adjusting the pH of the Tris-glycine buffer to a pH of 8.3 or 8.4. Doing so brings about a high load of chloride in the upper buffer chamber, and this causes the following effects (a) The protein separation will take much longer than expected, with some zones remaining poorly resolved (b) since chloride has a very high electrophoretic mobility compared to all other ions, only chloride ions will migrate towards the anode until there is no chloride remaining in the upper buffer chamber. Only after the depletion of chloride in the upper chamber will the protein ions start to migrate. Therefore the electrophoretic run will take several hours to overnight in a large format chamber (c) finally, the stacking will not be effective as in a perfect discontinuous buffer. The right procedure is to use only Tris-base, glycine, and SDS for the running buffer. The buffer should not be titrated even when pH is above 9 (1).

3.6. Over-focusing of IPG strips in 2-DE should be avoided

Two dimensional electrophoresis (2-DE) gels ideally show well-defined pattern of spots corresponding to all the individual proteins. However, the spots are sometimes blurred in certain areas. Vertical or horizontal streaks are seen instead of round spots. One reason for this is over-focusing. Of late, isoelectric focusing (the first dimension) mostly involves the use of polyacrylamide strips containing immobilized pH gradients. Since the pH gradient is fixed to the polyacrylamide matrix, it cannot go away owing to long exposure to an electric field. However, other disturbances in 2-DE maps can be brought about by over-focusing (1).

Some proteins are unstable, when they are at their isoelectric point for a certain time. It has been seen that some basic proteins are especially vulnerable to hydrolysis at their isoelectric point pH. This can bring about horizontal streaking from certain protein spots. Consequently, the focusing time applied to basic IPG strips should be limited to the minimum (1).

The addition of thiourea to urea, for protein extraction and IPG rehydration (16) results in increased solubility of hydrophobic proteins. As a result, there is a marked increase in protein spots in the 2-DE pattern compared with using urea alone. However, a strong vertical streak develops around pH 5.5 area in some cases. The protein spots along the acidic side of this streak appear more blurred than the spot pattern in the remaining areas of the gel. This phenomenon is not observed with all batches of thiourea. However, the phenomenon correlates also with the Volt hour load placed on an IPG strip. The amount of Volt hours placed on an IPG strip should be reduced if such an effect is observed (1).

3.7. PBS must be removed completely from cells prior to cell lysis

Cells derived from cell culture have to be washed efficiently with an iso-osmotic solution, such as phosphate buffered saline (PBS), prior to cell lysis to obtain antigens for high resolution two-dimensional electrophoresis. Sometimes, the PBS solution is not removed completely from the cells. Consequently, the salt contamination of the sample causes many and long horizontal streaks in the 2-DE electrophoresis pattern.

To correct this, the PBS must be removed from the cells with a pipette completely after the final wash. Care must be taken to see that not even a tiny droplet of PBS is left behind. To ensure this, it would be good to have one to three additional washes with 250 mM sucrose and 10 mM Tris-HCl (pH 7.0) (1).

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