COMPARISON OF THE EFFECTIVITY OF TWO DIFFERENT REHYDRATION SOLUTIONS ON THE SOLUBILIZATION OF PROTEINS SEPARATED BY TWO-DIMENSIONAL ELECTROPHORESIS

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Introduction

Two-dimensional gel electrophoresis (2-DE) is a widely used technique for the separation and characterization of proteins from complex biological samples. The first dimension separates proteins according to their relative charges. The second dimension separates proteins according to their apparent molecular weights. The use of immobilized pH gradients (IPG) in the first dimension enables a high reproducibility. Additional advantages of IPG include a high loading capacity and high resolution. This is true for soluble proteins (e.g. biological fluids) or for whole cell extracts where soluble proteins dominate, some problems are experienced when less soluble proteins are analyzed. In this case, quantitative protein losses were observed. This phenomenon has been attributed to insolubilization of proteins at, or very close to, their isoelectric point in the IPG matrix. Protein solubility in the first dimension and their transfer into the SDS gel could be improved by the proper combination of chaotropes and detergents in rehydration solution. Best results were obtained with denaturing solutions containing urea, thiourea and detergents (both nonionic and zwitterionic).

We analysed by 2-DE with IPG the possible effect of X-irradiation on the differentiation of human promyelocytic leukemia HL-60 cells towards monocytic lineage. The HL-60 cells were treated with 4(3-phorbol 12-myristate 13-acetate (PMA), X-ray irradiated and cultivated for additional three hours. To improve the sensitivity of our 2-DE approach, we tried to prefractionate HL-60 cells and used two different solutions for IPG rehydration. The aim of this study was to compare the influence of two various rehydration solutions on the solubility of proteins in tested mixtures.

Experimental part

Sample preparation

HL-60 cells were incubated for 72 hr with 100 nM-PMA and subsequently X-ray irradiated with 2Gy and incubated for 3 more hours. Afterwards cells were lysed in Nonidet P40 lysis buffer (20 mM-Tris-HCl, pH 8.0, 137 mM-NaCl, 10 % glycerol, 1 % Nonidet P40, 2 mM-EDTA, 50 mM-NaF) to which protease inhibitors (10 μg.ml⁻¹ leupeptin, 10 μg.ml⁻¹ aprotinin, 1 mM-PMSF) and phosphatase inhibitors were added and centrifuged (10 000 g, 15 min, 4 °C). Supernatants were recovered and frozen at -80 °C. The remaining pellet was resuspended in 10 volumes of urea lysis buffer (9 M urea, 2 % Nonidet P40, 3 % CHAPS, 70 mM-DTT, 50 mM-NaF) with protease inhibitors (1 μg.ml⁻¹ leupeptin, 1 μg.ml⁻¹ aprotinin, 1 mM-PMSF), phosphatase inhibitors (0.1 mM sodium orthovanadate) and 10 mM spermine base, centrifuged (200 000 g, 1 hr at ambient temperature) and supernatants were collected and stored as mentioned above.

2-DE electrophoresis

The determination of protein concentration was performed by Bicinchoninic acid protein assay kit (Sigma, Prague, Czech Republic). A commercial nonlinear immobilized pH 3-10 gradient (Pharmacia-Biotech, Uppsala, Sweden) was applied for the separation of proteins in the first dimension. The immobiline strips were rehydrated overnight in the standard rehydration solution: 8 M urea,
4% CHAPS, 10 mM-DTT, 1% carrier ampholytes (Reso-
yte pH 4-8) and a trace of bromophenol blue or in
urea/thiourea rehydration solution: 5 M urea, 2 M thiourea,
10 mM-DTT, 0.4% carrier ampholytes (Pharmalytes 3-10),
0.5% Triton X-100, 2% sulfobetaines (SB 3-10) (Ref.1)
and a trace of bromophenol blue. Samples containing 150
µg of proteins were loaded on immobiline strips. The 2-DE
with IEF and silver staining of separated proteins were
performed as described earlier1. Silver-stained gels were
scanned using a laser densitometer (4000 x 5000 pixels, 12
bits/pixel; Molecular Dynamics, Sunnyvale, USA) which
generated 30-megabyte images. This scanner was linked to
SunSPARC Workstation S20 (Sun Microsystems Inc.,
Mountain View, USA). The 2-DE image computer analysis
was carried out using MELANIE II package (Bio-Rad,
Richmond, USA).

Results and Discussion

To sensitize the 2-DE protein patterns and to find out
which sort of cellular proteins is affected by ionizing radi-
ation, we pre-fractionated HL-60 cell lysates into two sam-
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them, according to the co-migration with the purified protein, correspond to actin isoforms. This finding is in accordance with the anticipated effectivity of urea/thiourea rehydration solution for the solubilization of cytoskeletal proteins and with the presumed actin stress fibre changes in the radiation-treated cells, as well.

Finally, it can be stated that the application of urea/thiourea rehydration solution enriches the 2-DE protein patterns about groups of proteins that are missed using classical approaches. Since these proteins should belong to nuclear and cytoskeletal constituents which participate, e.g., in the development of anchorage independence and/or tumorigenic phenotypes the analysis of their expression is of the prime interest.

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An immediate advance of two-dimensional 2(DE) gel electrophoresis with immobilized pH gradients is the practical feasibility of setting up very narrow pH gradients in the most regions of the pH scale, with resultant dramatic improvement in resolution for some applications. However, when less soluble proteins are analyzed by this technique, quantitative protein losses are observed. It was shown that protein solubility could be improved by varying the detergents and chaotropes used for rehydration of the immobiline strips. Our results confirmed the high effectiveness of a modified urea/thiourea/sulfobetaines rehydration buffer in the solubilization of proteins occurring, especially, in acidic area of 2-DE gels in which the presence of cytoskeletal proteins can be anticipated. It was also found that proteins requiring a higher amount of chaotropic substances to enter immobiline strips could be detected using a standard rehydration solution and they are lost when the urea concentration is diminished.