

Ultrasensitive Detection of Unstained Proteins in Acrylamide Gels by Native UV Fluorescence

Jan Roegerer,[†] Petra Lutter,[‡] Ralf Reinhardt,[‡] Martin Blüggel,[‡] Helmut E. Meyer,^{‡,§} and Dario Anselmetti^{*†}

Bielefeld University, Universitaetsstrasse 25, 33615 Bielefeld, Germany, and Protagen AG, Emil-Fligge-Str. 76 A, 44227 Dortmund, Germany, and Ruhr University Bochum, Universitätsstrasse 150, 44801 Bochum, Germany

Visualization of proteins inside acrylamide and other gels usually relies on different staining methods. To omit the protein-staining procedure, we visualized unstained proteins inside acrylamide gels by laser excitation with ultraviolet (UV) light (280 nm, 35 mJ/cm²) and directly detected native UV fluorescence. In one-dimensional gels, a detection limit as low as 1 ng for bovine serum albumin and 5 ng for other proteins with a linear dynamic range (2.7 orders of magnitude) comparable to state of the art fluorescent dyes could be achieved. In addition, the application of this method to 20 µg of a whole cell lysate separated in a two-dimensional gel showed more than 600 spots. Since protein labeling always represents a serious obstacle in protein identification technologies, the working efficiency with our procedure can be considered as a significant improvement for protein visualization and reproducibility in proteomics.

Currently, two-dimensional gel electrophoresis (2-DE) represents the technology most widely used to separate complex protein mixtures for subsequent differential comparison (proteomics). Proteins are separated according to their isoelectric point (pI) in the first dimension and according to their apparent molecular mass in the second dimension. This method was first introduced by Klose and O'Farrell in 1975.^{1,2} Continuous improvements in mass spectrometry (MS) over the last 10 years routinely allow protein spot identification in 2-DE. For proteomics research, 2-DE gels of different states (e.g., healthy vs diseased states) are acquired and compared in order to investigate biochemical processes (e.g., disease-causing mechanisms). The image analysis of several dozens of gels is a bottleneck in proteomics because of limitations in reproducibility of 2-DE and staining processes.

Currently, different staining methods for the visualization of proteins in a gel have been established. Staining after separation: Silver staining is the most sensitive standard detection method (1 ng per band),³ but it is accompanied by problems, such as chemical modifications of the proteins.⁴ Furthermore, the staining and destaining procedures often result in a loss of protein and,

therefore, in a loss of sensitivity for mass spectrometrical analysis. Additionally, each protein has an individual staining behavior due to its compositional properties.⁵ Another drawback is the low dynamic range of this staining method. Staining with Coomassie Brilliant Blue G-250 (CBB) is widely used because it does not interfere with further MS analysis, however, at the cost of a lower detection sensitivity (20–60-fold).³ In contrast, labeling methods with fluorescent dyes are easier to handle and offer an improved dynamic range, but they are cost-intensive.⁶

Staining before separation: Labeling of proteins with fluorescent dyes before separation is a critical process, because the isoelectric point and the molecular mass of the proteins can be changed by this method as a result of the covalent modification. Radioactive labeling (¹⁴C, ³H) is the most sensitive method,³ but it is expensive, environmentally hazardous, and limited to biological experiments in which the organism can be incorporated with a radioactive medium (e.g., cell culture). Radioactive labeling prior to separation is possible because of little interference with the molecular mass and the isoelectric point. Both the experiment and the control can be compared within the same gel.⁷ A review of protein staining methods in electrophoresis is given by Williams.³

First attempts of protein detection in 2-DE by using UV light (280 nm) have been published recently. Yamamoto et al.⁸ detected UV absorption at 280 nm at a sensitivity limit of micrograms. Koutny et al. published a setup for native fluorescence detection in 1-DE gels during separation using a UV-lamp (253 nm) with a sensitivity of several micrograms per band.⁹ Kazmin et al.¹⁰ measured the fluorescence excited at 280 nm by shifting the emitted fluorescence of tryptophan into the visible region by a chemical modification using 20% trichloroacetic acid (TCA). They achieved a similar detection limit on the order of micrograms.

In this research paper, we present the first direct UV excitation and UV fluorescence detection of proteins in 2-DE at a very high sensitivity (1–5 ng).

* Corresponding author. Phone: +49-521 106 5391. Fax: +49-521 106 2959. E-mail: dario.anselmetti@physik.uni-bielefeld.de.

[†] Bielefeld University.

[‡] Protagen AG.

[§] Ruhr University.

(1) Klose, J. *Humangenetik*, **1975**, *26* (3), 231–243.

(2) O'Farrell, P. H. *J. Biol. Chem.* **1975**, *250* (10), 4007–4021.

(3) Williams, L. R. *Biotech. Histochem.* **2001**, *76* (3), 127–132.

(4) Scheler, C.; Lamer, S.; Pan, Z.; Li, X.P.; Salnikow, J.; Jungblut P. *Electrophoresis* **1998**, *19* (6), 918–927.

(5) Harry, J. L.; Wilkins, M. R.; Herbert, B. R.; Packer, N. H.; Gooley, A. A.; Williams, K. L. *Electrophoresis* **2000**, *21* (6), 1071–1081.

(6) Bumann, D.; Meyer, T. F.; Jungblut, P. R. *Proteomics* **2001**, *1* (4), 473–479.

(7) Monribot-Espagne, C.; Boucherie, H. *Proteomics* **2002**, *2*, 229–240.

(8) Yamamoto, H.; Nakatani, M.; Shinya, K.; Kim, B. H.; Kakuno, T. *Anal. Biochem.* **1990**, *191*, 58–64.

(9) Koutny, L. B.; Yeung, E. S. *Anal. Chem.* **1993**, *65*, 183–187.

(10) Kazmin D.; Edwards, R. A.; Turner, R. J.; Larson, E.; Starkey, J. *Anal. Biochem.* **2002**, *1*, 301 (1), 91–96.

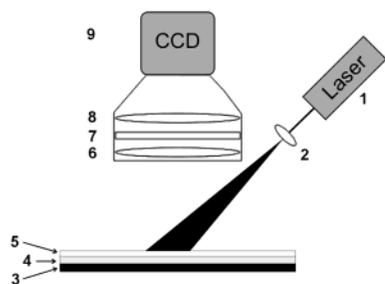


Figure 1. Experimental setup for UV detection of unstained proteins in gels: (1) UV laser, (2) spherical lens ($f = 100$ mm), (3) stainless steel plate, (4) acrylamide gel, (5) fused-silica window, (6) and (7) two Nikon lenses, (8) band-pass filter (300–375 nm), and (9) CCD camera.

MATERIALS AND METHODS

Electrophoresis and Staining. One-dimensional gel electrophoresis was performed on precasted 12% acrylamide gels in a tris-glycine–sodium dodecyl sulfate buffer system according to the manufacturer's instructions (Invitrogen, Karlsruhe, Germany). Separations of a four-protein mixture (carbonic anhydrase (bovine), 29 kDa; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (rabbit), 36 kDa; serum albumin (BSA) (bovine), 66 kDa; phosphorylase *b* (rabbit), 97 kDa; stock solution 100 $\mu\text{g}/\text{mL}$ of each protein) were run at different concentrations (1–500 ng/band) under reducing conditions.

Two-dimensional gel electrophoresis was performed as described¹¹ using a combination of carrier ampholyte isoelectric focusing (IEF) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). IEF was performed in rod gels containing 7 M urea and 2 M thiourea, 3.5% acrylamide, 0.3% piperazine diacrylamide, and a total of 4% carrier ampholytes, pH 2–11. Cellular proteins of EA.hy 926¹² whole cell lysates were prepared, and ~ 20 μg of protein was loaded at the anodic side of the IEF gels of 7 cm length and focused under nonequilibrium pH gradient electrophoresis (NEPHGE) conditions for 1842 Vh. SDS–PAGE was performed in gels containing 15% acrylamide using the IEF gels as stacking gels. The gel size was 70 \times 60 \times 1 mm³.

The gels were stored and fixed in 10% acetic acid, 50% ethyl alcohol, and 40% tridest water for 2 days to 3 weeks at 4 $^{\circ}\text{C}$. To reduce the background fluorescence, the gels were washed for 45 min in 50 mL of tridest water before the detection process and put on a 10 cm \times 10 cm stainless steel plate. The gel was covered by a fused-silica window to prevent drying and coiling up.

In addition, control gels were stained with silver¹³ and visualized using a white-light scanner (UMAX PowerLook 2100XL, Umax Systems GmbH, Willich, Germany).

Setup. The experimental setup¹⁴ was based on a UV excitation source and a UV detection system with a spectral range in the deep UV (laser, 240–300 nm; CCD, 190–1000 nm; fluorescence filter, 300–375 nm) (Figure 1). The excitation light was generated by a frequency tripled Ti:SA laser (Tsunami, Spectra Physics, U.S.A.) running at 840 nm, 80 MHz of 100-fs pulses creating a quasicontinuous wave (cw) light of 280 nm with 150 mW. The

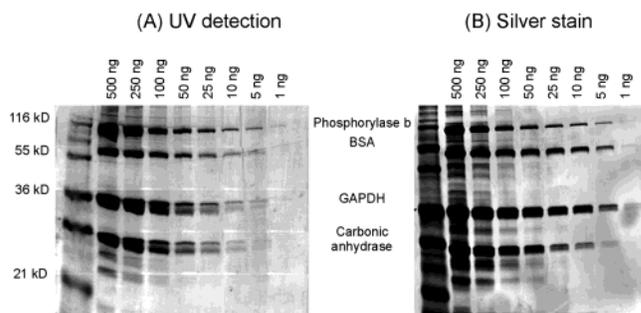


Figure 2. Comparison of UV detection and silver staining of proteins. A mixture of four proteins (carbonic anhydrase (29 kDa), GAPDH (36 kDa), BSA (66 kDa), and phosphorylase *b* (97.4 kDa)) was loaded onto two gels that were prepared identically. Proteins were detected with (A) UV detection and (B) silver stain following scanning (visible spectra). The proteins were loaded as follows: lane 1, molecular mass standard; lanes 2–9: 500, 250, 100, 50, 25, 10, 5, and 1 ng of each protein. UV exposure at 280 nm was 35 mJ/cm².

irradiation area was set to 1 cm² at 35 mW/cm² and imaged by two lenses (UV Nikkor, 105 mm, #4.5, Nikon) onto a UV-sensitive CCD camera (QE > 65%, Dyna Vision Lavision Biotec, Germany). To block the excitation light from the CCD camera, a UV band-pass filter (300–375 nm, Edmund Scientific, U.S.A.) was incorporated.

The maximum sample size was limited to 100 mm \times 100 mm (4 in \times 4 in). After each exposure (1 s), the sample was moved manually on a 1-cm spaced 10 \times 10 grid for sequential image acquisition. Thereafter, the images were arranged, corrected for uneven excitation intensities, and compressed to a resolution of 260 dpi by a commercial software (Davis 6.2, LaVision, Goettingen, Germany). The CCD dynamic range of 16 bit has been enlarged to a theoretical value of 22 bit by averaging over 64 pixels. In fluorescence detection, proteins give bright signals, and the background is dark. For better comparison with silver stained gels, all presented fluorescent images were digitally inverted.

RESULTS AND DISCUSSION

Four independent 1-DE gels with four different proteins in concentrations ranging from 1 to 500 ng/band were measured (Figure 2).

In all 1-DE gels, we observed a detection limit of 5 ng for each protein. For BSA, the detection limit was even lower, at 1 ng. Control gels with silver staining exhibited a detection limit of 5 ng for carbonic anhydrase and 1 ng for the other three proteins (GAPDH, BSA, Phosphorylase *b*).

In addition, two 2-DE gels (6 \times 7 cm²) loaded with 20 μg of protein (Figure 3) and two gels loaded with 40 μg protein were analyzed. In Figure 3, a comparison between an UV-analyzed 2-DE minigel (20 μg of protein) (A) with an identically prepared silver stained minigel (B) is presented. Spot detection with a commercial software (ImageMaster 2D Elite, Amersham Biosciences, Freiburg, Germany) yielded 600 and 700 protein spots for UV and silver staining, respectively. The difference in the total number of spots can be explained by the different sensitivity of the UV detection method in the present setup.

The reason for the low limit of UV detection is mainly based on the strong fluorescence from the aromatic amino acid tryptophan.

(11) Klose, J. *Methods Mol. Biol.* **1999**, *112*, 147–172.

(12) Edgell, C.-J. S. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 3734–3737.

(13) Heukeshoven, J.; Dernick, R. *Electrophoresis* **1988**, *9*, 28–32.

(14) Patent application CH 1344/02.

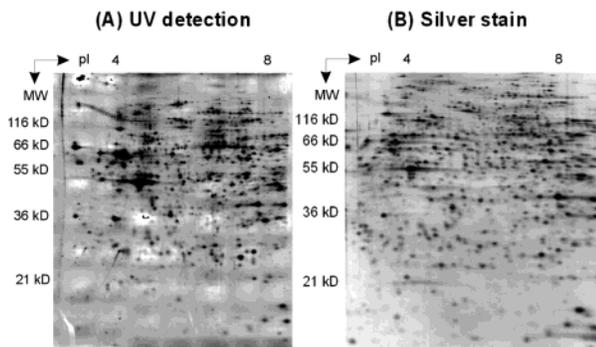


Figure 3. 2-DE spot pattern of EA.hy 926 whole-cell lysate. A 20- μ g portion of protein was loaded onto 2-DE gels with dimensions of 6×7 cm². (A) UV detection, inverse contrast representation (600 spots). The exposure was 35 mJ/cm². (B) Silver stain on identically prepared gel (700 spots).

tophan¹⁵ in combination with proper UV transparency and low background fluorescence from the gel. In 1DE gels, the protein fluorescence signal was always proportional to the protein amount, resulting in a linear dynamic range of 9 bit or 2.7 orders of magnitude (1–500 ng). This compares well with state-of-the-art fluorescent dyes.¹⁶

The sensitivity and dynamic range are limited by the background fluorescence of the gel, whereas photobleaching of the proteins represents only a minor limitation.

To discuss the limit of this method, we have to consider the fluorescence brightness of the involved amino acid side chains and their abundance in proteins. The fluorescence brightness of tryptophan is roughly 10 times stronger than tyrosine. Since smaller proteins are typically not present in a 2-DE gel, a database search on 1 026 890 proteins with molecular mass larger than 10 kD was performed (NCBI nr 9.23. 2002). Of all proteins larger than 10 kD, 90.5% contain at least one tryptophan and should be detected well. Another 9% contain at least one tyrosine, which will be detected at lower sensitivity. Only 0.5% contain neither tryptophan nor tyrosine (predominantly small proteins) and cannot be detected by this method.

We detected the proteins offline after separation. Imaging during separation could be distorted by the running buffer. The

fluorescence properties of a protein depend on the solvent as well as on pH. We performed SDS-PAGE followed by fixation and washing with tridest water, resulting in a gel at approximately neutral pH where some SDS may be left. The fluorescence background signal could not be reduced by further washing.

Direct UV detection of proteins offers many advantages, as compared to conventional staining methods, which result in a loss (modification) of the proteins for further analysis. The proteins can be detected without any staining and destaining. For this reason, modifications of the proteins can be excluded. Furthermore, a direct quantification of the protein amount is possible without staining-dependent variations of the signal intensities.

Compared to all staining methods, washing out of proteins is reduced to a minimum. Conclusively, more proteins in amount and type are available for MS. And last but not least, this method is faster, environmentally safe, and consumable cost-efficient.

CONCLUSION

In this paper, we present the first direct UV fluorescence detection of unstained proteins in gels at low-nanogram sensitivity. Image acquisition due to direct UV fluorescence detection of proteins omits the staining procedure and represents, therefore, a promising alternative for routine laboratory work.

Although the laser used in this investigation is an expensive part of the setup, the use of a UV lamp instead can make the setup more cost efficient.⁹

Major efforts have been made toward the automation of gel image analysis and comparison on one hand and of spot excision and preparation for mass spectrometric analysis on the other. But still, little attention has been paid to the automation of the staining and image capture process. The combination of a stainless spot detection and an automated spot excision would be another milestone in the automation process in proteomics.

ACKNOWLEDGMENT

This work has been supported by the Federal Ministry of Education and Research (BMBF) and the project "SCREEN". We acknowledge technical support from Stephan Woermer, Harald Schulz, and Gerd Wiebusch.

(15) Lakowicz, J. R. *Protein Fluorescence, Principles of Fluorescence Spectroscopy*; Plenum Publishing Corporation: New York, 1983; pp 342–342.

(16) Lopez, M. F.; Berggren, K.; Chernokalskaya, E.; Lazarev, A.; Robinson, M.; Patton, W. F. *Electrophoresis* **2000**, *21* (17), 3673–3683.