Giovanni Candiano[1*]
Maurizio Bruschi[1*]
Luca Musante[1]
Laura Santucci[1]
Gian Marco Ghiggeri[1]
Barbara Carnemolla[2]
Paola Orecchia[2]
Luciano Zardi[3]
Pier Giorgio Righetti[4]

[1]Laboratory on Physiopathology of Uremia, G. Gaslini Children's Hospital, Genova, Italy
[2]National Cancer Research Institute, Genova, Italy
[3]Institute G. Gaslini Chldren's Hospital, Genova, Italy
[4]University of Verona, Department of Agricultural and Industrial Biotechnologies, Verona, Italy

Blue silver: A very sensitive colloidal Coomassie G-250 staining for proteome analysis

A modified Neuhoff's colloidal Coomassie Blue G-250 stain is reported, dubbed "blue silver" on account of its considerably higher sensitivity, approaching the one of conventional silver staining. The main modifications, as compared to Neuhoff's protocol, were: a 20% increment in dye concentration (from 0.1% up to 0.12%) and a much higher level of phosphoric acid in the recipe (from 2% up to 10%). The "blue silver" exhibits a much faster dye uptake (80% during the first hour of coloration, *vs.* none with a commercial preparation from Sigma). Even at equilibrium (24 h staining), the "blue silver" exhibits a much higher sensitivity than all other recipes, approaching (but lower than) the one of the classical silver stain. Measurements of stain sensitivity after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of bovine serum albumin (BSA) gave a detection limit (signal-to-noise ratio >3) of 1 ng in a single zone. The somewhat lower sensitivity of "blue silver" as compared to classical silvering protocols in the presence of aldehydes is amply compensated for by its full compatibility with mass spectrometry of eluted polypeptide chains, after a two-dimensional map analysis, thus confirming that no dye is covalently bound (or permanently modifies) to any residue in the proteinaceous material. It is believed that the higher level of phosphoric acid in the recipe, thus its lower final pH, helps in protonating the last dissociated residues of Asp and Glu in the polypeptide coils, thus greatly favoring ionic anchoring of dye molecules to the protein moiety. Such a binding, though, must be followed by considerable hydrophobic association with the aromatic and hydrophobic residues along the polypeptide backbone.

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1 Introduction

Countless staining procedures have been reported in the literature ever since the pioneering work of Ornstein and Davis [1, 2] established a high-resolution zone electrophoretic method in polyacrylamide gels, called disc-electrophoresis on account of several discontinuities established along the migration path for sharpening the analyte zones. They can be roughly divided into the following categories: (i) organic dyes; (ii) silver stains; (iii) negative stains; (iv) fluorescent stains. In the latter category, perhaps the dyes that have emerged in daily practice are the SYPRO family, such as SYPRO red and SYPRO orange [3, 4], or SYPRO Tangerine [5] reported to interact with SDS-protein complexes with sensitivity levels of the order of 1.0–4.0 ng/mm^2. More recently, for 1-D (SDS-PAGE) or 2-D (IEF-SDS) gels, a SYPRO Ruby stain has been reported [6], which provides a sensitive, gentle, fluorescence-based method for detecting proteins. SYPRO Ruby is a permanent stain composed of ruthenium as part of an organic complex that interacts noncovalently with proteins. Stained proteins can be excited by UV light of about 302 nm or with visible light of *ca*. 470 nm. Fluorescence emission of the dye is approximately 618 nm. The detection sensitivity of SYPRO Ruby (0.5–1 ng protein/mm^2) is superior to that of Amido black, Coomassie Blue and india ink staining and nearly matches colloidal gold staining. An additional advantage of SYPRO Ruby is that it is fully compatible with subsequent protein analysis, such as Edman-based sequencing and mass spectrometry.

Another series of methods for protein detection are the negative or reverse stains, which are generally based on the formation of insoluble metal salts, leaving protein bands unstained when viewed against a dark back-

Correspondence: Prof. P. G. Righetti, Department of Agricultural and Industrial Biotechnologies, University of Verona, Strada le Grazie No. 15, I-37134 Verona, Italy
E-mail: righetti@sci.univr.it
Fax: +39-45-8027929

Abbreviations: IAA, iodoacetamide; **TBP**, tributylphosphine

* Giovanni Candiano and Maurizio Bruschi contributed equally to the work

ground. They act rapidly (15 min), and allow easy recovery of proteins from gel matrices, since they do not require a fixation step. Some commonly used negative stains, with their detection limits, include: copper chloride (5 ng/mm) [7], zinc chloride (10–12 ng) [8], potassium acetate (0.12–1.5 μg) [9], and sodium acetate (0.1 $\mu g/mm^3$) [10]. While most of the negative stains require the presence of detergent in the gel for achieving protein visualization, Candiano *et al.* [11] have described a negative stain acting both in the presence and absence of SDS, based on precipitation of methyl trichloroacetate, able to detect as little as 0.5 ng of protein.

Perhaps, however, the most popular classes of stains are the organic dyes and silvering protocols, due to their ease of use, simple protocols, and high sensitivities. When first introduced in 1979 by Merril *et al.* [12], the silver stain was reported to increase the sensitivity of protein detection, over Coomassie Blue staining, by 2000-fold, from tenths of a microgram to tenths of a nanogram. All silver stain protocols depend on the reduction of ionic to metallic silver onto the protein surface. Currently, the three main silver stains used for protein detection on polyacrylamide gels are: diamine or ammoniacal stains [12, 13]; nondiamine, silver nitrate stains [14], and silver stains based on photodevelopment [15]. There exist also variegated silver stains, producing not just the monochromatic brown or black colors, but also bluish, yellowish or reddish hues, due to diffractive light scattering of light by microscopic silver grains having sizes below 100 nm in diameter [16, 17].

While it cannot be denied that silvering is decidedly the most sensitive stain so far reported, most scientists working on quantitative, differential proteome analysis prefer classical organic dyes of the Coomassie family. This is due to the fact that their lower sensitivity is largely compensated by the stain reproducibility, due to insensitivity to a number of parameters (temperature, quality of solvents, developing times) which render silvering protocols highly erratic. Moreover, a large number of silvering techniques do not allow for further processing of proteins, since aldehydes irreversibly cross-link polypeptide chains, although aldehyde-free silver protocols have been recently reported, albeit with lower sensitivities [18].

Coomassie stains, ever since first reported in 1963 by Fazekas de St. Groth [19], have always enjoyed a widespread popularity, due to their ease of use and reasonable sensitivity (*ca.* 0.5 $\mu g/mm^2$). One mg protein can bind 0.17 mg of Amido black, 0.23 mg of Fast Green, 1.2 mg of Coomassie Blue R-250 (R = reddish hue) and 1.4 mg of Coomassie Blue G-250 (G = greenish hue) [20]. This relatively high staining intensity of Coomassie stains is apparently due to dye-dye interactions among Coomassie dyes that are ionically bound to, or in hydrophobic association with, the protein molecules [20]. Basic amino acids appear to be important for the interaction between the sulfonated Coomassie moieties and the protein surface, as shown by the correlation between the intensity of Coomassie staining and the number of Lys, His, and Arg residues in the protein [21]. Further evidence comes from the observation that polypeptides rich in Lys and Arg are aggregated by Coomassie G dye molecules [22]. A quantum jump came with the work of Neuhoff *et al.* [24] who described staining with Coomassie in a micellar state, first obtained in presence of sulfosalicylic acid [23], and subsequently by addition of phosphoric acid. The latter method seems to have the highest sensitivity of all Coomassie staining variants described (*ca*. 30 ng per band) but takes at least overnight.

In an attempt to ameliorate this unique staining protocol [24], we describe here a modified Neuhoff procedure reaching a sensitivity as high as 1 ng protein/band, thus in the range of the most sensitive silver stains, aptly nicknamed “blue silver”. It is here shown that such a staining protocol is fully compatible with 2-D maps in proteome analysis and subsequent mass spectrometry of eluted polypeptide chains.

2 Materials and methods

2.1 Reagents

Urea, thiourea, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), iodoacetamide (IAA), tributylphosphine (TBP), all Immobilines and sodium dodecyl sulfate (SDS) were obtained from Fluka Chemie (Buchs, Switzerland). Tris(hydroxymethyl)aminomethane, tributyl phosphate, and DL-dithiothreitol (DTT) were from Sigma (St. Louis, MO, USA). All Ampholines, bromophenol blue, and agarose were from Pharmacia-LKB (Uppsala, Sweden). Acrylamide, *N,N*’-methylenebisacrylamide, Coomassie Brilliant Blue G-250 and *N,N,N’,N’*-tetramethylethylenediamine (TEMED) were from Bio-Rad Labs (Richmond, CA, USA). Trichloroacetic acid (TCA), acetone, and methanol were from Merck (Darmstadt, Germany).

2.2 Preparation of blue silver micellar solution

Whereas the classical Neuhoff dye recipe contains the following (final concentrations in the ready-to-use solution): 2% H_3PO_4, 10% $(NH_4)_2SO_4$, 20% methanol, and 0.1% Coomassie G-250, we have tested ample variations of concentrations of most ingredients in the mixture, while keeping the dye concentration fixed at a 20% higher level

(0.12%) as compared with Neuhoff's recipe. The final concentrations adopted in the working colloidal "blue silver" solution were: 0.12% dye, 10% ammonium sulfate, 10% phosphoric acid, and 20% methanol. This produced a dark green dye solution, which turned to a deep blue when adsorbed onto the polypeptide chains fixed in the polyacrylamide gel, or blotted onto membranes. The dye solution is prepared as follows, by sequentially adding the various ingredients as here indicated: to a water solution (1/10 of the final volume) the desired amount of phosphoric acid is added, so that, in the final volume, its concentration will be 10%; to this, add the required amount of ammonium sulfate (in powder), calculated to obtain a final concentration of 10%. When the ammonium sulfate has dissolved, add enough Coomassie Blue G-250 (in powder) to obtain a final concentration of 0.12%. When all solids have dissolved, add water to 80% of the final volume. To this solution, under stirring, add anhydrous methanol to reach a 20% final concentration. This stock dye solution should be kept in a brown bottle and is stable at room temperature for $>$ 6 months.

2.3 Human kidney tubular epithelial cells

Human kidney tubular epithelial cells were developed as already described [25]. They were grown under humidified atmosphere of 5% CO_2 at 37°C in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 1% nonessential amino acids, 1% L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. Cells at confluence were lysed with 5 mM phosphate buffer at pH 8.0, 1 mM EDTA, plus phosphatase and protease inhibitors. After 30 min at 4°C, the mixture was centrifuged at 14 000 rpm for 15 min and the supernatant was immediately frozen in liquid nitrogen prior to storage at −80°C. Cytosolic extracts of human kidney tubular epithelial cells were reduced, alkylated, and delipidated, as illustrated below, prior to isoelectric focusing.

2.4 Solubilization and preparation of delipidated epithelial cells for 2-D PAGE

The protein content of the cytosolic epithelial cells preparation was adjusted to 5 μg/μL, after addition of a 10% SDS, 3% DTT, 40 mM Tris, and 0.1 mM EDTA solution and treated for 5 min at 100°C. The sample thus solubilized was delipidated and cleaned with a solution consisting of tri-*n*-butylphosphate:acetone:methanol (1:12:1), cooled in ice [26]. Fourteen mL of this mixture were added to the SDS-solubilized cytosolic epithelial cells to a final acetone concentration of 80% and incubated at 4°C for 90 min. The precipitate was pelleted by centrifugation at 2800 × *g* for 20 min at 4°C. After washing with the same delipidizing solution, it was centrifuged again and then air-dried. The pellet was finally dissolved in the focusing solution, *i.e.*, 7 M urea, 2 M thiourea, 4% w/v CHAPS, 5 mM TBP, 20 mM IAA, 40 mM Tris, and 0.1 mM EDTA, pH 8.8. Prior to IEF, the sample was let to incubate in this solution for 3 h, so as to allow for proper reduction and alkylation [27]. Excess IAA was destroyed by adding an equimolar amount of DTT, so as to prevent overalkylation during the focusing step. Amount loaded per IPG strip: 100 μg protein.

2.5 Rehydration of IPG strips and 2-D electrophoresis

All these steps were performed precisely as described previously [28]. At the end of the separation, proteins were visualized by three different protocols: a commercial Sigma Neuhoff-like recipe, a freshly prepared Neuhoff stain [24], and the micellar Coomassie G-250 solution here described.

2.6 In-gel enzymatic digestion

Following the staining procedure, the 2-D gel was washed in water and the spots of interest were excised. The gel discs containing the protein of interest were washed twice with 50% ACN + 50% ammonium bicarbonate (5 mM solution) for a minimum of 2–3 h untill full decoloration of the gel. After that, wash twice (10 min each time) in 100% ACN. In-gel digestion was carried out in 100 mM ammonium bicarbonate, 1 mM $CaCl_2$ pH 8.9, 30% ACN, and 12.5 ng/μL (1 μg) sequencing grade modified trypsin (Promega, Madison, WI, USA) overnight at 37°C. After tryptic digestion, the sample was dried in a vacuum centrifuge (Savant Instruments, Farmingdale, NY, USA) to reduce ACN concentration, diluted in 0.25% formic acid and filtered using a 0.02 μm Anodisc 13 filter (Whatman) in a MicroFilter system (Protein Solutions, Lakewood, NJ, USA).

2.7 LC/ESI-MS/MS analysis of tryptic peptides

An automated LCQ-DECA MS/MS ion trap mass spectrometer coupled to a HPLC Surveyor (ThermoFinnigan) and equipped with a 1 × 150 mm column, Vydac C_{18}, 5 μm, 300 Å (Dionex Company, San Francisco, CA, USA) was used. Peptides were eluted from the column using an ACN gradient, 5% B for 3 min followed by 5 to 90% B within 52 min (eluent A: 0.25% formic acid in water; eluent B: 0.25% formic acid in ACN) at a flow-rate of 50 μL/min. The capillary of the ion trap was kept at 200°C and the voltage at 30 V. Spray voltage was 5.0 kV. Spectra

were acquired in automated MS/MS mode: each full MS scan (in the range 400–2000 *m/z*) was followed by three MS/MS of the most abundant ions, using a relative collision energy of 35%. Computer analysis of peptide MS/MS spectra was performed using Version 1.2 of the TurboSEQUEST software (University of Washington, licensed to ThermoFinnigan) and searched against the National Center for Biotechnology Information (NCBI) human protein database.

3 Results

Figure 1 shows a kinetic study of dye uptake. It can be appreciated that the slowest dye adsorption occurs in the Sigma preparation, whereas the fastest coloration occurs with the present recipe. In fact, in the case of our colloidal dye, 80% dye uptake occurs in the first hour of staining, *vs.* 40% of the Neuhoff and essentially nihil for the commercial dye. Moreover, the final equilibrium uptake (> 24 h) is the highest in the case of our protocol.

When taking albumin alone, after a monodimensional SDS-PAGE, it can be appreciated, from Fig. 2, that the main band (M_r 66.200) is visible down to a concentration of *ca.* 1 ng. Since, by visual inspection, it is always quite difficult to reach objective conclusions, the above statement has been reinforced by a series of scans of each track, presented in Fig. 3. If one takes as a sensitivity limit a signal-to-noise ratio (S/N) of 3, as customarily done, it can be seen that the 1 ng detection limit is quite reasonable. In fact, a minute peak can also be appreciated at somewhat lower loads (0.75 ng) but definitely not at a load of 0.1 ng.

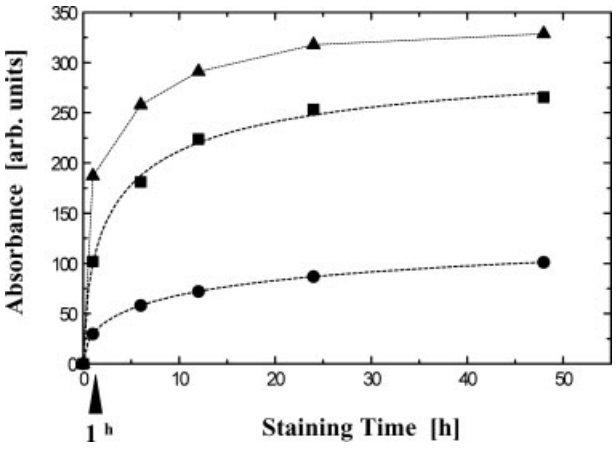

Figure 1. Kinetics of dye uptake (absorbance *vs.* time) from the three different stain recipes. (▲) "blue silver"; (■) Neuhoff's stain; (●) commercial Sigma preparation.

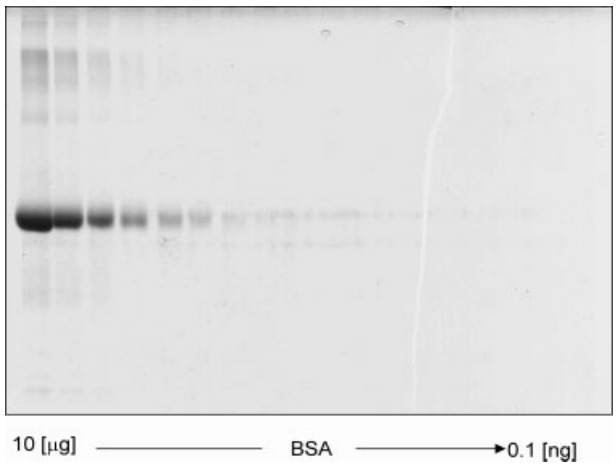

Figure 2. Sensitivity of blue silver upon SDS-PAGE of BSA. Stain uptake down to 1 ng protein can be appreciated.

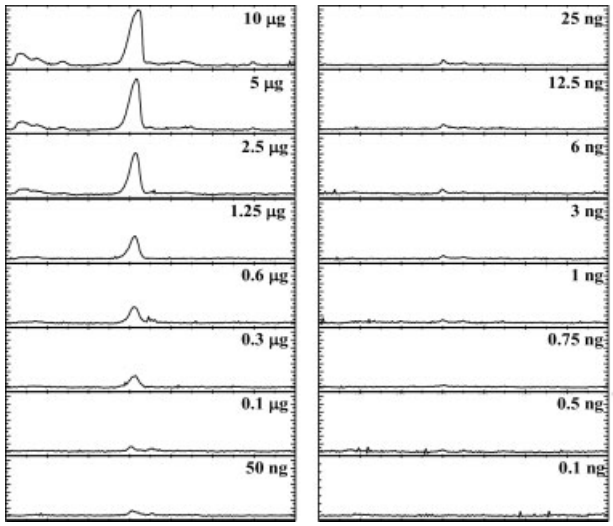

Figure 3. Densitometric evaluation of stain uptake by BSA after SDS-PAGE. The gel of Fig. 2 was subjected to scanning at 600 nm. A good signal-to-noise ratio (> 3) is evident down to 1 ng protein load.

Figure 4 shows a part of a 2-D map of human kidney tubular epithelial cells, stained, from left to right, with the Neuhoff's, silver and the new "blue silver" stain. It can be appreciated that our staining protocol is decidedly closer to the sensitivity of a classical silver than to the original Neuhoff. The somewhat lower sensitivity, however, is amply compensated by the fact that all protein spots, once eluted, are fully compatible with mass spectrometric analysis. In the present high-sensitivity silver stain, this is not possible since, in order to obtain such very high sensitivity, a classical silver protocol, utilizing glutaraldehyde, had to be adopted. Figure 5 shows a human kidney tubular epithelial cells 2-D map, stained with "blue silver". All

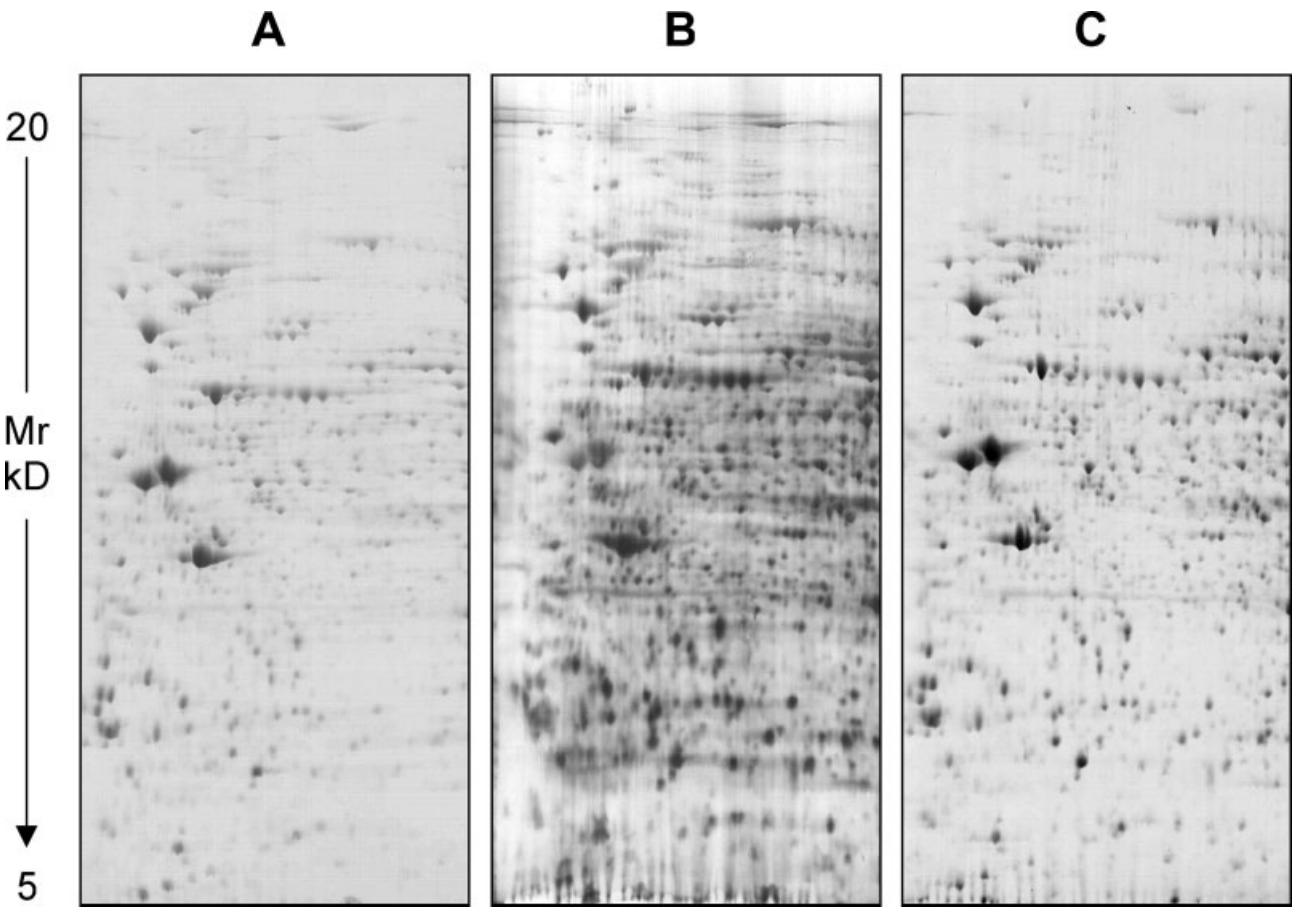

Figure 4. Comparison of stain sensitivity after a 2-D map of human kidney tubular epithelial cells of the three stains tested (A = Neuhoff, B = silver stained, C = blue silver). The three panels show an identical part of the 2-D map. In all cases the total protein load was 100 μg.

the spots marked by numbers have been eluted, analyzed by MS and identified, as shown in Table 1. It is thus demonstrated that our protocol is fully compatible with MS analysis and that even fainter spots can produce a viable signal, leading to protein identification in data bases.

4 Discussion

The present report describes a variant of Neuhoff stain [24], by which the sensitivity of micellar Coomassie G-250 is further improved so as to approach that of silver protocols. Two major modifications were made: (i) to increase the total amount of dye by 20%, from 0.1% to 0.12% in the final recipe; (ii) to substantially augment the concentration of phosphoric acid, from the original 2% up to 10%. Even higher concentrations of the acid were tried, up to 20%: with these progressively higher amounts, the micelles of Coomassie, prior to the addition of methanol, tended to assume a brownish to reddish hue, indicating a marked change in size. Interestingly, as the amount of phosphoric acid was progressively increased, the dye uptake by the protein zones became even faster and the spots assumed a deeper blue coloration. However, the polyacrylamide gel matrix also became intensively stained, requiring extremely long destaining procedures. Nevertheless, the fact that even higher amounts of phosphoric acid tended to stain the proteins even more intensely indicates that this ingredient plays a fundamental process in dye uptake by the protein surface. One reason for that could be the increased acidity of the solution: in the Neuhoff protocol, the final pH of the dye concoction is 1.20, whereas in our recipe (10% H_3PO_4) the pH was 0.50 (in 20% phosphoric acid the final pH would be 0.02). It is quite possible that these additional pH decrements play a role in protonating the few remaining Glu and Asp residues still partially dissociated, as well as the carboxy terminal, thus lowering and in fact almost abolishing any negative charge along the polypeptide coil. At these low

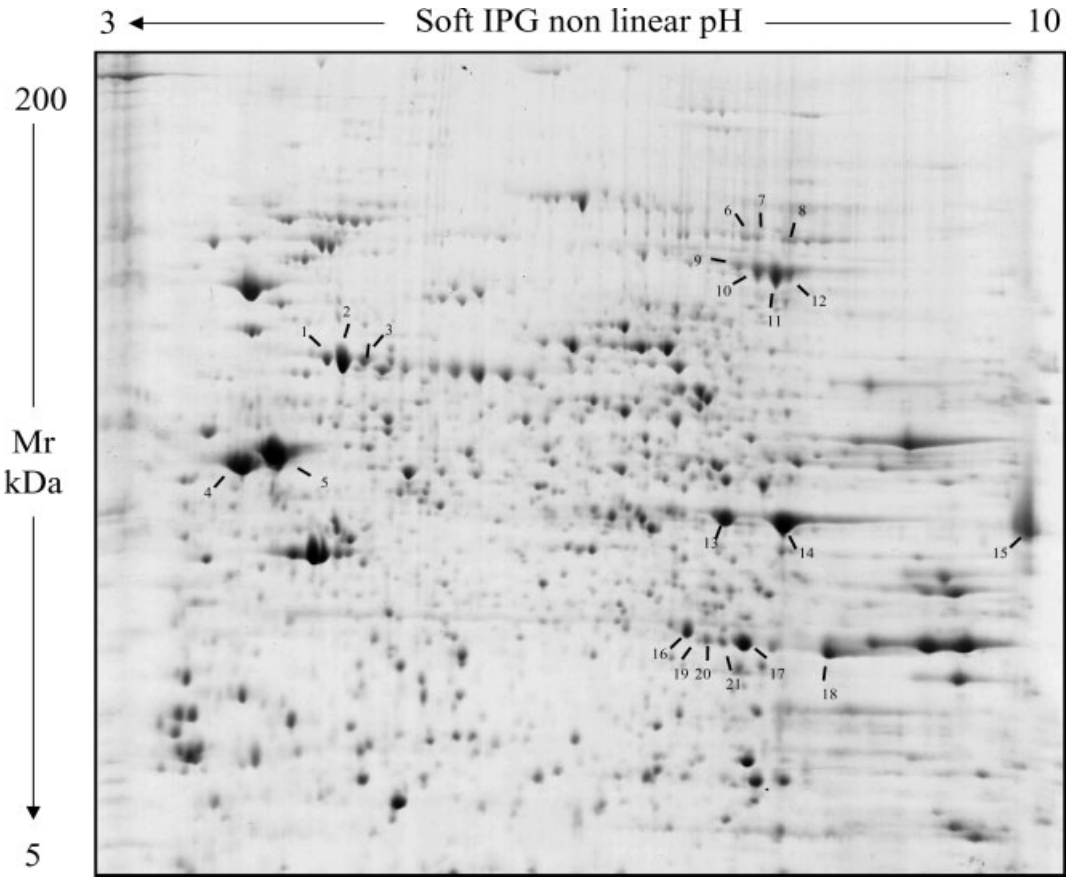

Figure 5. 2-D map of cytosolic extract of human kidney tubular epithelial cells, run in a 3–10 nonlinear IPG strip made from dilute polyacrylamide (3.3%T). The spots marked with the Nos. 1–21 were eluted and identified by mass spectrometry.

Table 1. Identified proteins of cytosolic extract of human kidney tubular epithelial cells

Spot No.	Accession No.[a)]	Protein	Sequence coverage (%)
1, 2, 3	P11142	Heat shock cognate 71 kDa protein	60
4	P07437	Tubulin β-1 chain	78.7
5	P08670	Vimentin	95
6, 7, 8	gi 284156	Heterogeneous ribonuclear particle	49.4
9, 10, 11, 12	P13639	Elongation factor 2	80.9
13, 14	P06733	α-Enolase	92
15	P04720	Elongation factor 1 α-1	59
16, 17, 18	P15121	Aldose reductase	83
19	P04083	Annexin 1	31.5
20, 21	P07355	Annexin 2	35

a) Accession numbers: SWISS-PROT database or gi number of NCBI database

pH values, though, the sulfonated residues of the dye would still be extensively dissociated, thus facilitating docking of the dye onto the protein surface *via* salt bridges with any available His, Lys, and Arg residue. Once the dye molecules have been anchored to the polypeptide coils *via* these ionic interactions, other types of linkages must occur, since it has been demonstrated, at least in the case of albumin, that, upon extensive binding

of Coomassie Blue G-250 (as induced at high molar ratios, dye:protein 20:1), the complex undergoes a hypsochromic shift, from 602 nm, at low saturation, down to 582 at maximum saturation [29]. This suggests that, in addition to initial ionic bonding, there must be another type of interaction, of substantial hydrophobicity in nature. The organization of planar ring systems, with extensive conjugation of π-orbitals, may explain these observations [30, 31]. In fact Silber and Davitt [29] have calculated that nonionic binding forces could account for as much as 58% of the total binding energy, when the requirement for a lengthy peptide is fulfilled. It has been additionally demonstrated that, at acidic pH values, and at high molar ratios (dye:protein 20:1) as many as 100 dye-binding sites would be available along the denatured BSA random coil. Assuming just one dye molecule bound per each site, this would amount to a total of 100 Coomassie Blue molecules bound to BSA, *i.e.*, one Coomassie, on the average, every six amino acid residues, quite a high surface density of the dye, indeed [29]. If this is what occurs in the case of our "blue silver protocol", this would very well explain the substantial increment in sensitivity.

Conversely, not much is gained when varying the final concentration of methanol in the "blue silver" recipe. In fact, at any level of methanol above 20% (we have tested concentrations up to 30%), the colloidal micelles seem to disintegrate and the dye looses sensitivity. Thus, it would appear that the 20% level adopted in most recipes reaches just the right balance in hydrophilicity/hydrophobicity of the final solution so as to favor formation of colloidal dye micelles and their fast uptake by the denatured protein surface.

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