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A method for in-gel fluorescent visualization of proteins after native and sodium dodecyl sulfate polyacrylamide gel electrophoresis

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ABSTRACT

We have developed a simple one-step 30-min method for fluorescent visualization of proteins in native and sodium dodecyl sulfate polyacrylamide gel electrophoresis (PAGE) gels. The method is based on formation of strong fluorophores via potassium ferricyanide-provoked oxidation of tryptophan (Trp). Following PAGE, gels are soaked in water solution of potassium ferricyanide (100 mM) and NaOH (1 M) and are kept in the dark for 30 min. Gels are then transferred to water and scanned. The sensitivity of the method was slightly lower compared with standard Coomassie Brilliant Blue (CBB) staining. The method can be useful when rapid acquisition of data is of the essence. After preview, gels can be post-stained using the CBB protocol for further analysis. The intensity of fluorescence is dependent on Trp number, so the protocol might find application in the quantification of Trp residues as illustrated here. Importantly, there is room for improvement of the method. Namely, according to excitation–emission matrix analysis of stained protein bands, maximal fluorescence intensity (at 345/460 nm) was 3.5-fold higher compared with the settings that were available on a commercial imager (395/525 nm). As a supplement, we present an upgrade of the previously described method for in-gel detection of non-heme iron-binding proteins that also employs potassium ferricyanide.

Fast, simple, and sensitive methods for in-gel visualization of proteins following native and sodium dodecyl sulfate (SDS)[1] polyacrylamide gel electrophoresis (PAGE) are needed in order to increase efficiency, particularly in multistep experiments. The standard Coomassie Brilliant Blue (CBB) procedure is lengthy, whereas shorter CBB protocols (processing times: 20 min to 1 h) require excessive gel handling and might produce a sordid smell [1–4]. Native fluorescence of proteins that is based on constituent fluorophores, such as tryptophan (Trp), has been employed for visualization of proteins since the early days of electrophoresis [5,6]. The detection of native fluorescence has shown significant advances since but still requires nonstandard approaches such as laser excitation and microelectrophoresis [7]. Nevertheless, Trp appears to be a good starting point for generation of stronger fluorophores via chemical modifications. For example, a staining method has been developed using ultraviolet-induced reactions of Trp with trihalo compounds (trichloroethanol, chloroform, and trichloroacetic acid). Fluorescent products of these reactions emit sufficiently in the visible region to visualize protein bands with sensitivity that is comparable to the CBB protocol [4,8]. Furthermore, it has been shown that very strong fluorophores can be produced via Trp oxidation [9,10]. The method has been applied for quantification of free Trp in biological samples by thin-layer chromatography (TLC) with fluorescence detection. Excitation was in the 320- to 420-nm range (maximum at 365 nm), with emission in the 400- to 550-nm range (maximum at 450 nm) [10].

Here, we have developed a method for fluorescent visualization of gel bands that is based on oxidation of Trp in proteins. In the TLC study, Kato and coworkers [10] oxidized Trp with potassium ferricyanide under high pH and heat. The latter setting might not be needed because potassium ferricyanide has been shown to react with Trp residues in proteins at room temperature; high pH ($\geqslant$10.0) is still required [11]. Trp residues are highly hydrophobic, and as such they are usually buried within the protein structure [12]. Taking this into account, as well as the requirement for high pH, NaOH-provoked protein denaturation appeared to be a useful tactic in making Trp more accessible to the reaction with ferricyanide. Besides this, we present a modified protocol for staining of non-heme iron-binding proteins that also involves ferricyanide.

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[1] *Abbreviations used:* SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; CBB, Coomassie Brilliant Blue; Trp, tryptophan; TLC, thin-layer chromatography; BSA, bovine serum albumin; MnSOD, manganese superoxide dismutase.

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Materials and methods

PAGE and staining protocol

PAGE was carried out on a Mini-PROTEAN Tetra Cell electrophoresis system with PowerPac HC power supply (Bio-Rad Laboratories, Life Science Group, Hercules, CA, USA) under nondenaturing/denaturing conditions in gels (1 mm thickness) containing 8% running and 4% stacking polyacrylamide gel. A constant current of 15 mA/25 mA per gel was applied. Electrophoresis buffers and gels were prepared as described previously [13]. Samples for SDS PAGE were denatured by heating in buffer containing 0.7% (m/v) SDS and 1.7% (m/v) 2-mercaptoethanol. Two types of samples were loaded on gels: bovine serum albumin (BSA) and human plasma, which was obtained from the blood of healthy volunteers as described previously [14]. Bromophenol blue marker was omitted because it shows fluorescence. Following electrophoresis, different staining procedures and scanning modes were systematically tested for sensitivity. $K_3[Fe(CN)_6]$ (100 or 200 mM) was applied for 15 min, 30 min or 1 h simultaneously with or after NaOH (1 M; 30-min or 1-h incubation period). It is important to note that $K_3[Fe(CN)_6]$ is light sensitive, so the solutions were prepared fresh for staining and kept in the dark. The performance of $K_3[Fe(CN)_6]$ (100 mM) was also tested in the water or in the presence of HCl (3 M; denaturing concentration). This procedure did not result in the formation of visible bands. In addition, we tested some other oxidizing agents/mixtures such as $KMnO_4$ (1, 25, or 100 mM), H_2O_2 (2%), combinations of H_2O_2 (0.4 or 20 mM) and the redox-active metals copper (10 mM) and ferrous iron (0.2 mM), and the combination of iron (0.2 mM) and ascorbate (10 mM). In most of these procedures, some bands were observed, but the intensity of fluorescence was always significantly lower compared with the best procedure employing $K_3[Fe(CN)_6]$. Finally, the effect of heating (100 °C/3 min [10]) on $K_3[Fe(CN)_6]$/NaOH staining was tested, but it resulted in the annihilation of fluorescence. The intensity of fluorescence of Trp shows a maximum at pH values near 10.0 [15]. Pertinent to this, two post-staining solutions, water (pH following gel soaking was > 10.0) and Tris buffer (pH 10.0), were tested. There was no difference in resulting sensitivity. All chemicals were obtained from Sigma–Aldrich (St. Louis, MO, USA). Gels were scanned using an Ettan Dige Imager (GE Healthcare, Little Chalfont, UK) with five different scanning modes (combinations of excitation and emission filters). The best results were obtained using excitation at 395 nm (bandpass: 20 nm) and emission at 525 nm (bandpass: 25 nm); other settings were as follows: pixel size, 40 μm; exposure, 2. In all other modes, excitation and emission were at higher wavelengths. All images here were obtained via only one scan of the gel. For comparison purposes, gels obtained on the same experimental day were also stained using CBB protocol as follows. After washing three times with water for 1 min, the gels were treated for 20 min with the solution of acetic acid (10%) and methanol (50%). Gels were transferred to the same solution also containing CBB R-250 (0.1%) for 20 min. Destaining of gels for clear background was performed by incubation in methanol (5%) and acetic acid (7%) for 4 h to overnight.

Testing the application of the staining protocol in Trp quantification

Native PAGE of five proteins showing different numbers of Trp per subunit—myoglobin from equine heart (2 Trp, mass 17,083 Da, UniProt entry P68082), carbonic anhydrase I from human erythrocytes (6 Trp, 28,870 Da, P00915), manganese superoxide dismutase (MnSOD) from *E. coli* (6 Trp, 23,097 kDa, P00448), carbonic anhydrase II from bovine erythrocytes (7 Trp, 29,246 kDa, P00918), and ascorbate oxidase from *Cucurbita* sp. (14 Trp, 61,794 Da, P37064)—was performed using the same settings as described in the previous section. All proteins were purchased from Sigma–Aldrich except MnSOD, which was a kind gift from Mihajlo Spasić at the Institute for Biological Research, University of Belgrade. Proteins were loaded at equimolar (subunit) amounts (10 μl of 20 μM solution, i.e., 0.2 nmol/lane), corresponding to (in μg of proteins/lane) 3.42 (myoglobin), 5.77 (carbonic anhydrase I), 4.62 (MnSOD), 5.85 (carbonic anhydrase II), and 12.36 (ascorbate oxidase). Gels were stained using our method or the CBB protocol. The intensities of bands were quantified using ImageJ (National Institutes of Health). For this purpose, the grayscale coloring of images was inverted (in Adobe Photoshop CS4). Experiments were conducted on 3 experimental days in pairs (potassium ferricyanide- and CBB-stained gels). Mean values of band intensity (± standard deviations) were scaled to nanomoles (nmol) of Trp per lane and to micrograms (μg) of proteins per lane, respectively, and a linear fit was performed (OriginPro 8, OriginLab, Northampton, MA, USA). This fit was used as a calibration curve in the next experiment. Aliquots from the stock of unknown (in order to mimic a realistic biological sample) concentration of ovalbumin (3 Trp, 44,287 Da, P01012) were applied each day on both gels. Band intensities were measured, and the numbers of nmol of Trp and μg of proteins were established from calibration curves.

Excitation–emission matrix fluorometry of stained in-gel BSA

Following native PAGE (250 μg of BSA/lane was loaded) and ferricyanide staining, an area of gel with BSA band was cleaved and placed in a solid sample holder. Fluorescence spectra were acquired using Fluorolog FL3-221 with a 450-mW Xe lamp (Jobin Yvon Horiba, Paris, France) and FluorEssence 3.5 software (Horiba Scientific, Kyoto, Japan) and the following settings: FrontFace mode (45°); excitation range, 300 to 500 nm; emission range, 300 to 700 nm; increment, 5 nm; slit (bandpass), 4 nm. The emission detector signal was scaled by reference quantum counter signal (S1/R1). A piece of stained protein-free gel of the same area was used as blank.

Development of method for non-heme iron-binding protein detection

Leong and coworkers [16] developed a simple method for in-gel detection of non-heme iron-binding proteins such as ferritin (with a 1-μg limit) and transferrin (with a 100-μg limit). Following PAGE, gels are incubated in 50 mM Tris–HCl buffer (pH 7.5) with $K_3[Fe(CN)_6]$ (100 mM) and NaCl (100 mM) for 10 min in the dark. After that, gels are transferred to trichloroacetic acid (10%)/methanol (10%) water solution, where bands of iron-binding proteins develop blue color [16]. This is based on the reaction of ferricyanide with ferric (or ferrous) iron that results in the formation of an insoluble pigment (Prussian blue) [17]. Here, we tested a set of procedures for preincubation of native PAGE gels with Fe^{3+} ($FeCl_3$) in order to increase the sensitivity of the method. Tested $FeCl_3$ concentrations were 0.5, 1, and 5 mM, in the presence of NaCl (50, 100, and 136 mM), with 15-, 30-, and 60-min incubation times. Following staining, gels were washed once with water and scanned using a flatbed scanner.

Results and discussion

We found that the most sensitive ferricyanide staining procedure for in-gel fluorescent visualization of proteins is to incubate the gel in freshly prepared water solution of $K_3[Fe(CN)_6]$ (100 mM) and NaOH (1 M) for 30 min in the dark. Of note, a short exposure to light did not have negative effects on sensitivity. After this, the gel is transferred to water and scanned. Excitation was at