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Visible fluorescent detection of proteins in polyacrylamide gels without staining

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Abstract

2,2,2-Trichloroethanol (TCE) incorporated into polyacrylamide gels before polymerization provides fluorescent visible detection of proteins in less than 5 min of total processing time. The tryptophans in proteins undergo an ultraviolet light-induced reaction with trihalocompounds to produce fluorescence in the visible range so that the protein bands can be visualized on a 300-nm transilluminator. In a previous study trichloroacetic acid or chloroform was used to stain polyacrylamide gel electrophoresis (PAGE) gels for protein visualization. This study shows that placing TCE in the gel before electrophoresis can eliminate the staining step. The gel is removed from the electrophoresis apparatus and placed on a transilluminator and then the protein bands develop their fluorescence in less than 5 min. In addition to being rapid this visualization method provides detection of 0.2 μg of typical globular proteins, which for some proteins is slightly more sensitive than the standard Coomassie brilliant blue (CBB) method. Integral membrane proteins, which do not stain well with CBB, are visualized well with the TCE in-gel method. After TCE in-gel visualization the same gel can then be CBB stained, allowing for complementary detection of proteins. In addition, visualization with TCE in the gel is compatible with two-dimensional PAGE, native PAGE, Western blotting, and autoradiography.

Keywords: Protein visualization; Fluorescence; Tryptophan; 2,2,2-Trichloroethanol

Quick and sensitive protein visualization methods are needed for PAGE, since they will give increased efficiency for high-throughput detection. A wide selection of protein visualizations are available (reviewed in [1,2]). However, a sensitive method that would allow rapid detection without excessive handling of the gel, which may cause tears, would be beneficial. Recently a rapid method for visualizing proteins on polyacrylamide gels based on the fluorescence of modified tryptophans has been reported [3]. The results presented here significantly improve upon this technique.

New visualization methods are typically accessed by comparison to CBB[1] staining [2]. Although standard CBB methods take several hours to stain and destain, rapid CBB methods that visualize proteins with 20 min of processing time have been developed; however, these methods involve heating, consume staining and destaining solutions, and can produce objectionable odors [4]. The sensitivity limit of CBB is in the submicrogram range [2], but the limit changes for different proteins because proteins destain to different extents. There are many variations to the standard CBB method to allow for low background and increased sensitivity [5,6]. A modified rapid Fairbanks Coomassie stain gave a sensitivity limit of 5 ng [4]. A significant improvement to the CBB technique is colloidal Coomassie blue staining, which requires no destaining and is sensitive to the 8- to 10-ng range [2,7]. Furthermore an improved CBB G-250 technique allows detection of 0.5 ng of protein [8]. The modified CBB techniques providing exceptional sensitivity are not in common usage. Silver staining is 100 times more sensitive than CBB with its limit in the nanogram range [2,9], but this method is much more labor intensive.

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[1] *Abbreviations used:* CBB, Coomassie brilliant blue; TCE, 2,2,2-trichloroethanol; TCA, trichloroacetate; 2-DE, two-dimensional electrophoresis; IPG, immobilized pH gradient; RMSD, root mean square deviation; HRP, horseradish peroxidase.

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For reproducible results timed steps must be followed and constant reaction temperatures used.

Many fluorescent dyes have been developed for protein detection [2]. The linear dynamic range of SYPRO Ruby Protein Gel stain is 1–1000 ng, which gives a range over three orders of magnitude [10]. This range is much larger than those for standard CBB and silver staining methods [2]. In addition the sensitivity limit for the SYPRO Ruby method is 1–2 ng [10]. Staining with SYPRO Ruby requires 4 h but it is an end point stain so the time is not critical [10]. Also SYPRO Ruby, like most protein visualization methods, is biased by amino acid content. The mechanism of binding for SYPRO Ruby is similar to the CBB mechanism where binding to proteins is dependent on lysine, arginine, and histidine residues [2]. As a result it could be expected that proteins not efficiently visualized with CBB will not bind SYPRO Ruby also. There are also fluorescent covalent labeling methods. Covalent labeling methods derivatize either cysteine or lysine residues [2]. Most systems derivatize the sample before electrophoresis, thereby possibly affecting mobility in SDS–PAGE. For example 2-methoxy-2,4-diphenyl-3(2H)-furanone, a lysine-reactive label, provides a sensitivity limit of 1 ng and changes both the isoelectic point and the molecular weight of a protein [11].

A novel technique to fluorescently visualize proteins in polyacrylamide gels with trichloroacetic acid and chloroform was recently published [3] and Zhong et al. [12] have already made use of this efficient staining technique. In this staining method the gel was soaked in a trihalocompound, which was chloroform or trichloroacetic acid, and then illuminated on a 300-nm UV transilluminator to produce fluorescent protein bands. The fluorescent product of the light-induced reaction with chloroform has been proposed to add a formyl group to the indole ring of tryptophan [13]. Thus visualizing proteins with trihalocompounds is dependent on tryptophan content. In this study the speed and sensitivity of utilizing tryptophan photomodification for protein visualization has been greatly improved by incorporating the halocompound TCE into the gel before polymerization so that post-electrophoresis soaking is not required. By this method 0.2 μg of typical globular proteins can be visualized in less than 5 min. For carbonic anhydrase, which has a higher percentage of tryptophan than typical globular proteins, as little as 20 ng of protein can be detected. TCE in-gel visualization is complimentary to CBB visualization because the same gel can then be CBB stained.

Materials and methods

SDS–PAGE and staining methods

Low-molecular-weight standards were separated on a 12% SDS–PAGE 1-mm thick gel as per the standard Laemmli method using the Protean II gel system from Bio-Rad Laboratories (Hercules, CA, USA) or the Atto Electrophoresis (AE-6450) Dual Mini Slab system (Atto Corp.). The low-molecular-weight standards from Bio-Rad Laboratories, contained phosphorylase b (97 kDa, 2.3% Trp), bovine serum albumin (66 kDa, 0.8% Trp), ovalbumin (45 kDa, 1.3% Trp), carbonic anhydrase (31 kDa, 4.5% Trp), trypsin inhibitor (21 kDa, 1.8% Trp), and lysozyme (14 kDa, 7.85% Trp). All tryptophan percentages are calculated as percentage weight. Pharmacia low-molecular-weight electrophoresis calibration kit standards contained the same proteins as those from Bio-Rad except lysozyme, which was replaced with α-lactalbumin (14.4 kDa, 3.2% Trp). Pharmacia standards explicitly state the amount of each protein present. Other purified protein samples loaded were EmrE, DmsD:His6, and TehB:His6 from *Escherichia coli*. EmrE was purified according to Winstone et al. [14]. DmsD:His6 [15] and TehB:His6 [16] were purified with a nickel agarose column followed by size-exclusion chromatography.

Protein detection was done using CBB or the novel techniques presented. For the CBB staining a modified Fairbanks method [17] where both 0.05% CBB R250 and 0.05% of the colloidal form CBB G250 are used along with 0.1% cupric acetate. The gel is stained overnight followed by 5 h destaining. Two different methods were used to visualize proteins with TCE (Aldrich). In the first method TCE, usually at 0.5% (v/v), was incorporated into the gel. TCE was dissolved in the gel buffer and then acrylamide, SDS, ammonium persulfate, and TEMED were added as usual. The stacking gel was prepared as usual. The gel was then visualized as described below. In optimization studies 0.02, 0.05, 0.1, 0.2, 0.5, 1.0, and 2.0% TCE (v/v) were added to the gel before polymerization. To optimize the UV exposure time an image of a gel visualized with TCE in the gel was taken after 30 s, 1, 2, 5, 10, and 15 min of irradiation and the intensities of the 1-μg bands were compared. For comparison a second method where TCE was soaked into the gel post-electrophoresis was used. The 12% SDS–PAGE gel was run and then soaked in 10% TCE (v/v) in water:methanol (1:1) for 10 min. Then the gel was washed in water and visualized as described below.

Protein visualization

Proteins are visualized by placing the gel on the UV transilluminator and irradiating the gel for 2–5 min, during which time the protein bands become visible as bluish-green bands against a pale blue background of the gel matrix. A 300-nm UltraLum Electronic UV transilluminator with a Cohu high-performance monochrome charge-coupled device camera from Rose Scientific (Edmonton, AB, CAN) was used to take photographs of the gel. Picture frames are averaged to increase the intensity of the picture and to better capture protein bands. In the

images shown 4–50 frames are summed. Pixel intensity of bands was evaluated using Scion Image V1.62 software (ftp:zippy.nimh.nih.gov). Two ways were used to measure intensity. In the first way the density of the background above and below a band is averaged and the density of the band is subtracted from this, giving the intensity of the band. In the second way the image is inverted and a density profile is plotted for each lane from the gel. The area under each peak is calculated using the trapezoid rule to give the intensity.

To calculate the intensities for Fig. 3, the sum of the intensities of phosphorylase b, albumin, ovalbumin, and trypsin bands was used. These were chosen because they contain percentages of tryptophan near the average for soluble proteins. The calculated weight percent of tryptophan in a general set of proteins from eukaryotes and prokaryotes is 2.24% [18]. Four sets of data were used for Fig. 3 and averaged.

Statistical analysis

The accuracy and precision of TCE in-gel visualization and CBB visualization were quantified by calculating the RMSD. Two sets of data for the six proteins in the Pharmacia standards were used. For each protein, linear regression of intensity versus protein mass for four points was used to predict the protein mass of the approximately 1-μg band, which had been excluded from the linear regression. The difference between the actual mass and the calculated mass for the protein was used to calculate the RMSD as a measure of accuracy. For precision the same procedure was used except that the average mass calculated from the intensity was used instead of the actual mass to calculate RMSD.

Western blot analysis

Immediately after visualizing the 12% SDS–PAGE gel with 0.5% TCE, it was electroblotted to nitrocellulose. The blot was then blocked overnight with 5% milk in Tris-buffered saline. The blot was then incubated with the primary antibody, antiHis6 (Cedarlane Laboratories Ltd.), and developed with antimouse horseradish peroxidase (HRP) conjugate and HRP conjugate substrate kit from Bio-Rad laboratories.

Native PAGE

A 12% native PAGE, free of SDS in all buffers with 0.5% TCE added before polymerizing the gel, was ran. Protein visualization was done as described above.

Two-dimensional electrophoresis

For 2-DE analysis, membrane-free *E. coli* extract was prepared as follows. A 250-mL Luria–Bertani media culture of HB101 was grown by 1% inoculation from an overnight culture to an O.D. of 0.6. This was then French pressed at 16 kpsi with two passes in the presence of 2 mM phenylmethylsulfonyl fluoride (Sigma) and 0.1 mg/ml DNaseI (Sigma). Then a low-speed spin at 2700*g* was performed followed by a high-speed spin at 346,000*g*. Protein concentration was then determined with a Lowry assay. For isoelectric focusing 250 μg of membrane-free extract was loaded onto an 11-cm pH 3–10 immobilized pH gradient (IPG) Immobiline DryStrip (Amersham Biosciences) at the anodic end with a sample cup using the MultiphorII system (Amersham Biosciences). Focusing was conducted for 50.4 kV-h at a maximum of 3500 V. The IPG strip was then equilibrated in equilibration solution (20% glycerol, 6 M urea, 50 mM Tris, pH 8.8, and 2% SDS) with 2% dithiothreitol (Bio-Rad) for 15 min and then in equilibration solution with 2.5% iodoacetamide (Sigma) for 15 min. The second dimension was run by placing the IPG strip on a 1-mm-thick continuous 12% SDS–PAGE containing 0.5% TCE and overlayed with 1% agarose in SDS–PAGE buffer with 0.002% bromophenol blue. The gel was run with a mini-Protean III at 200 V for 45 min (Bio-Rad). The images were acquired in the same way as was done for one-dimensional SDS–PAGE.

Results and discussion

Comparison with Coomassie brilliant blue

Visualization with TCE–UV-modified tryptophan is more sensitive than CBB and faster. Fig. 1 shows a 12% SDS–PAGE gel loaded with Bio-Rad low-molecular-weight standards detected with 0.5% TCE in the gel (Fig. 1A) compared to CBB staining. The same gel visualized with the TCE in-gel technique is then stained with CBB (Fig. 1B). Comparison of the TCE in-gel method (Fig. 1A) with the CBB method (Fig. 1B or C) shows that TCE staining is slightly more sensitive than CBB staining. For example the 97- and 31-kDa bands in lane 1 are more intense when using the TCE in-gel method. The recommended amount to load for the low-molecular-weight standards for Coomassie staining is 0.5 μg, but half that amount is sufficient for the TCE in-gel method. The presence of TCE in-gels during electrophoresis does not impair the mobility of the proteins, as can be seen by comparing a CBB-stained gel containing 0.5% TCE (Fig. 1B) to one without TCE (Fig. 1C), because the protein bands are not shifted. The method used for (Fig. 1D) employed soaking the gel with 10% TCE and the bands are almost as intense as when TCE was put into the gel, but adding TCE to the gel before polymerization significantly decreases the processing time (see Figs. 1A and D). After completion of the running of a PAGE gel, detection of the proteins

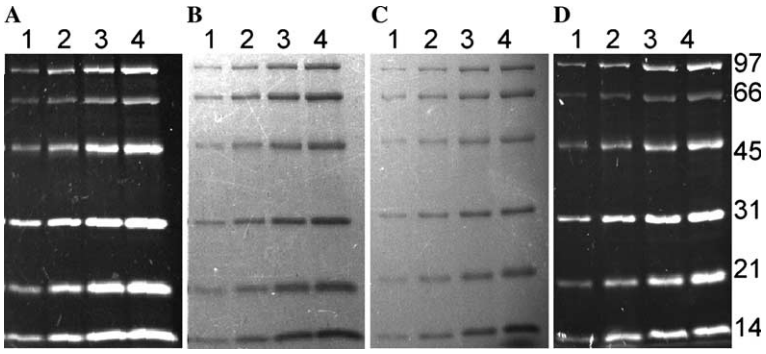

Fig. 1. Comparison of the TCE in-gel visualization method to CBB staining. (A) 0.5% TCE was incorporated into the gel. (B) CBB stain of the same gel from A. (C) CBB stain of a gel free of TCE. (D) 10% TCE was used a post-electrophoresis stain. On all frames the lane numbers 1, 2, 3, and 4 denote 0.25, 0.5, 1, and 1.5 μg per band of Bio-Rad low-molecular-weight standards.

is obtained in a total time of less than 5 min. Visualization using TCE in the gel is the preferable method because of the speed at which results can be obtained and it is slightly more sensitive than CBB. Furthermore, less handling of the gel means fewer accidents.

Statistical analysis was done to investigate the accuracy of quantifying protein bands and the reproducibility of visualizing protein bands using the TCE in-gel technique. Protein bands from 12% SDS–PAGE gels with 0.5% TCE in the gel were quantified, from both the fluorescent visualization and the CBB visualization method. The RMSD from the actual mass of protein in the 1-μg band was 0.24 μg for the TCE method and 0.07 μg for the CBB method. Thus the CBB method was more accurate. Precison as measured by the RMSD from the average mass was 0.18 μg in the TCE method and 0.15 μg in the CBB method. Thus the reproducibility for both methods is about the same.

Optimization of technique

Initially different combinations of TCE, trichloroacetic acid, and chloroform were tried for post-electrophoresis staining. TCE or combinations of TCE and TCA were found to give the greatest intensity (results not shown). Pairs of halocompounds with different polarities were investigated and it was shown that they do not complement each other by modifying tryptophans in various environments. The amount needed for optimum protein detection was found to be 10% TCE in 1:1 methanol:water (results not shown). Putting TCE in the gel gave slightly greater intensity and was faster (see Fig. 1). Also less TCE is used, therefore reducing waste.

For Bio-Rad protein standards, adding more TCE to the gel (up to about 0.5% TCE) increases the band intensity after which the intensity no longer increases (results not shown). Thus the optimum amount of TCE to use is 0.5% of the separating gel volume.

The standard procedure for visualization in this method is to expose the gel for 2 min. The intensity increases rapidly for the first 2 min of UV irradiation. After 2 min of irradiation the intensity remains fairly constant up to 10 min, after which it decreases slowly at a rate of less than 2% per minute (results not shown).

Properties of technique

All the proteins in the low-molecular-weight standards are detectable at 0.2 μg, for globular proteins with typical percentages of tryptophan (0.8–2.3%) (Fig. 2A). Some proteins are detectable at 20 ng because they have high tryptophan percentages (the 4.5% band in Fig. 2A). In Fig. 2, TCE in-gel visualization is presented both as the actual image (Fig. 2A) or the inverted image (Fig. 2B). The TCE in-gel detection method offers a linear dynamic range from 0.2 to 2 μg with a correlation coefficient of 0.99 (see Fig. 3). The upper limit of the linear dynamic range is lower with proteins of higher tryptophan percentage. Thus with high tryptophan content the dynamic range is shifted to lower protein amounts, for both the upper limit and the sensitivity limit. The linear dynamic range for specific proteins was also investigated with the TCE post-electrophoresis staining method and showed similar patterns for the linear dynamic range (results not shown).

The intensity of protein bands increases linearly as the mass of tryptophan increases, as shown in Fig. 4. TCE reacts with the excited indole ring of tryptophan under UV light to attach a functional group to the indole ring [13]. The modified tryptophan then emits a visible fluorescence when under UV light. Thus the fluorescence from the TCE in-gel visualization method is expected to be entirely a result of tryptophan residues. With regard to the mass of tryptophans in a protein band the limit of detection is 0.7 ng. The upper limit of the linear dynamic range is 100 ng of tryptophan per protein band.

TCE in-gel visualization will be especially useful for membrane proteins. An integral membrane protein,

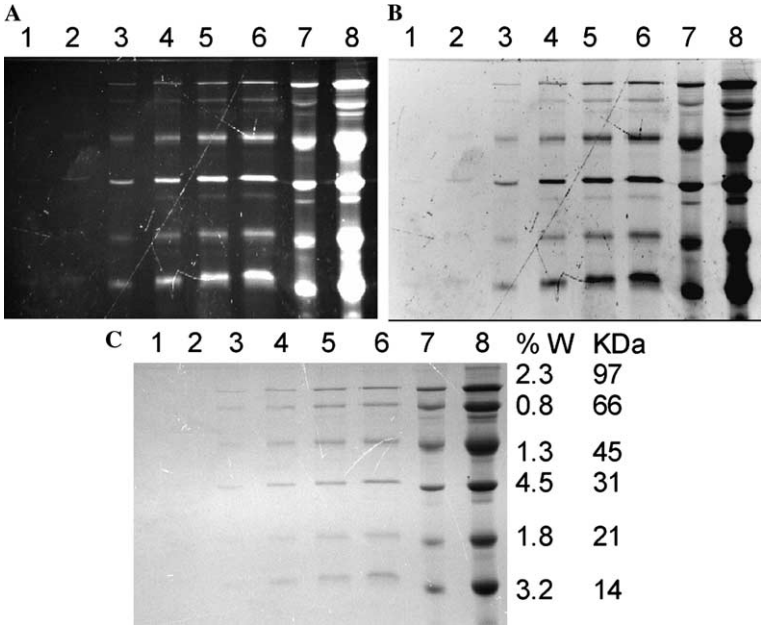

Fig. 2. All low-molecular-weight standard proteins are detectable at 0.2 μg with the TCE in-gel technique. (A) 0.5% TCE incorporated into a 12% SDS–PAGE gel. (B) Black and white inverted picture of A. (C) CBB stain of the same gel from A. The lane numbers 1–8 denote 0.02, 0.05, 0.2, 0.5, 1, 1.5, 2, and 5 μg per band, respectively, of Pharmacia low-molecular-weight standards. The percentage weight of tryptophan content (% W) and the molecular weight (kDa) are aligned with the respective protein.

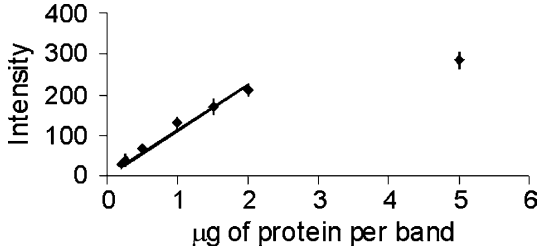

Fig. 3. Fluorescence intensity increases linearly with protein concentration. Intensities are from a 12% SDS–PAGE with 0.5% (v/v) TCE in the gel. The sum of intensities from phosphorylase b, bovine serum albumin, ovalbumin, and trypsin inhibitor with 0.2, 0.25, 0.5, 1.0, 1.5, 2.0, or 5.0 μg per band. The error bars are the standard deviations. The correlation coefficient for the line including all points up to 2 μg per band is 0.99.

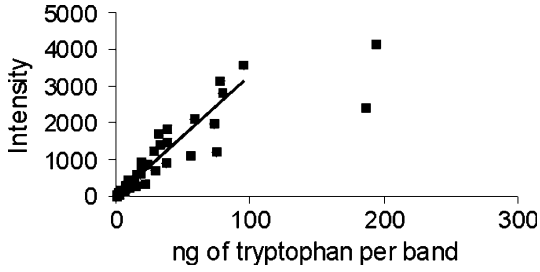

Fig. 4. Fluorescence intensity increases linearly with the mass of tryptophan in a protein band. Intensities were obtained from the SDS–PAGE gel shown in Fig. 2. The nanogram of tryptophan was calculated for all protein bands and plotted versus the intensity. Linear regression was done omitting two points with over 100 ng of tryptophan. The correlation coefficient is 0.93.

EmrE (5.0% Trp), is barely visible at 0.5 μg when stained with CBB (Fig. 5A, lane 2), but the TCE in-gel technique gives very intense bands at 0.5 μg and is even highly visible at 0.25 μg (Fig. 5A, lane 1). DmsD (8.7% Trp), a peripheral membrane protein, is visualized with TCE better than CBB (Fig. 5B, lanes 1 and 2). Furthermore the sensitivity limit for detecting DmsD is 10 ng (results not shown). TehB, a soluble protein, appears to be visualized with equal intensity by both methods (Fig. 5B, lanes 3 and 4).

Additional TCE in-gel applications

This new technique allows for visualization of proteins before Western blotting or autoradiography. Fig. 5B shows proteins visualized by the TCE in-gel method (lanes 1–4) followed by a Western blot of the His tags on DmsD and TehB. This will allow for confirmation that an appropriate protein pattern is seen before performing a Western blot procedure. To obtain a CBB stain of the protein pattern a separate gel would need to be run. In addition this demonstrates that TCE in the gel does not hinder transfer of proteins to nitrocellulose. Similarly this method can be used to visualize proteins before autoradiography, adding considerable speed to such experiments (results not shown).

The TCE in-gel visualization is also applicable to native PAGE. Fig. 5C shows a 12% native PAGE of low-molecular-weight standards and DmsD visualized with 0.5% TCE in the gel. This gives the same protein pattern as that seen in a gel free of TCE visualized with CBB (Fig. 5D).

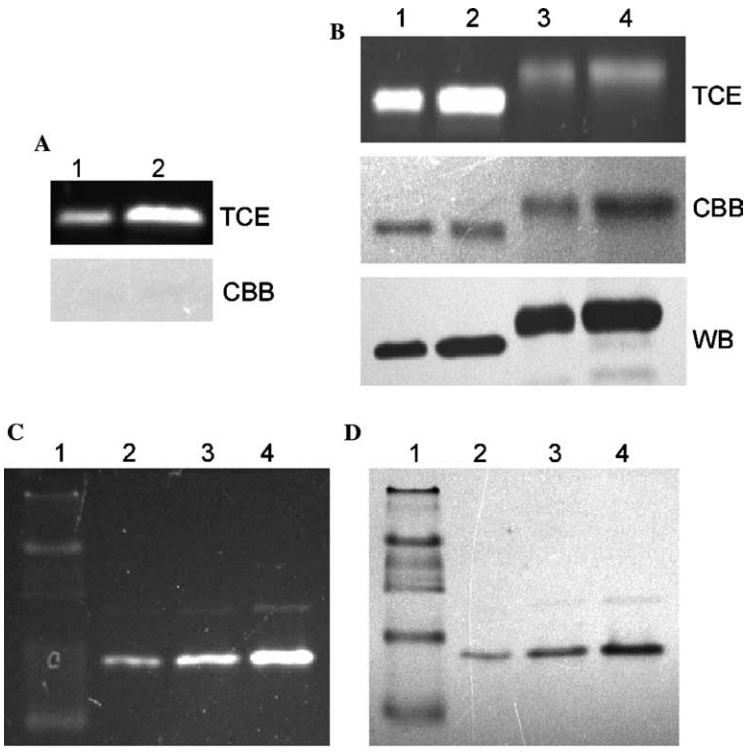

Fig. 5. TCE in-gel technique is useful for visualization of membrane proteins, before Western blotting (WB) and for native PAGE. (A) SDS–PAGE of an integral membrane protein, EmrE, 0.25 and 0.5 μg (lanes 1 and 2), visualized with TCE in the gel, followed by CBB staining. (B) SDS–PAGE of DmsD:His6, 0.25 and 0.5 μg (lanes 1 and 2) and TehB:His6 (lanes 3 and 4). The gel was visualized with the TCE in-gel method and then a Western blot and probing with antiHis6 were performed. A duplicate gel was TCE in-gel visualized and then CBB stained. (C) TCE in-gel visualization of 12% native PAGE with low-molecular-weight standards (lane 1) and DmsD:His6, 0.25, 0.5, and 1 μg (lanes 2, 3, and 4). (D) CBB stain of a duplicate of the 12% native PAGE gel in B without TCE.

Of particular interest to proteomic endeavors is the use of TCE in-gel visualization to provide a rapid method for visualizing 2-DE gels. A 2-DE gel of *E. coli* extract visualized with 0.5% TCE in the second dimension is shown in Fig. 6A. In Fig. 6B an inverted picture of the TCE in-gel method is shown for easier comparison with the CBB-stained gel. In addition to being rapid, the fluorescent pattern allows images of lesser or greater intensity to be obtained by changing the number of frames that are summed. With a less intense image it is possible to separate spots, which are blurred together on a CBB-stained gel. With a more intense image it is possible to identify weaker spots.

Comparison of the spots visualized by the TCE in-gel technique to those by the CBB technique shows that most of the spots visualized by CBB are visualized by TCE. Although some spots are not visualized by the TCE in-gel method that are visualized by CBB, there are some spots that TCE visualizes adequately that CBB visualizes poorly. To compare visualization patterns given by the two methods, a representative area is blown up and displayed in Fig. 6D from an inverted TCE image and in Fig. 6E after CBB staining. In this area there are two spots (S1 and S4) that are not visualized by TCE and there are two spots (S2 and S3) very poorly visualized by CBB that can be seen in the TCE visualization. Visualization with TCE is thus complimentary to CBB, since initial visualization with TCE detects spots that might be missed by CBB staining. Any spots missed by TCE visualization can be detected by subsequent CBB staining of the same gel.

Concluding remarks

This study demonstrates an improved technique for fluorescence visualization of proteins by tryptophan photomodification that does not require any post-electrophoresis manipulation. It has been demonstrated that placing TCE into the gel before polymerization allows for rapid protein detection, which is more sensitive than the standard CBB. The method presented has improved upon the fluorescence visualization techniques presented by Kazmin et al. [3]. TCE in-gel visualization allows for a safer and faster technique. TCE is less volatile than chloroform and thus less likely to be inhaled. Although the TCE in-gel method is less sensitive than the most sensitive SYPRO method, it is much faster and far less expensive.

TCE–UV-modified tryptophan protein detection has the potential to be especially beneficial for detection of integral membrane proteins because the

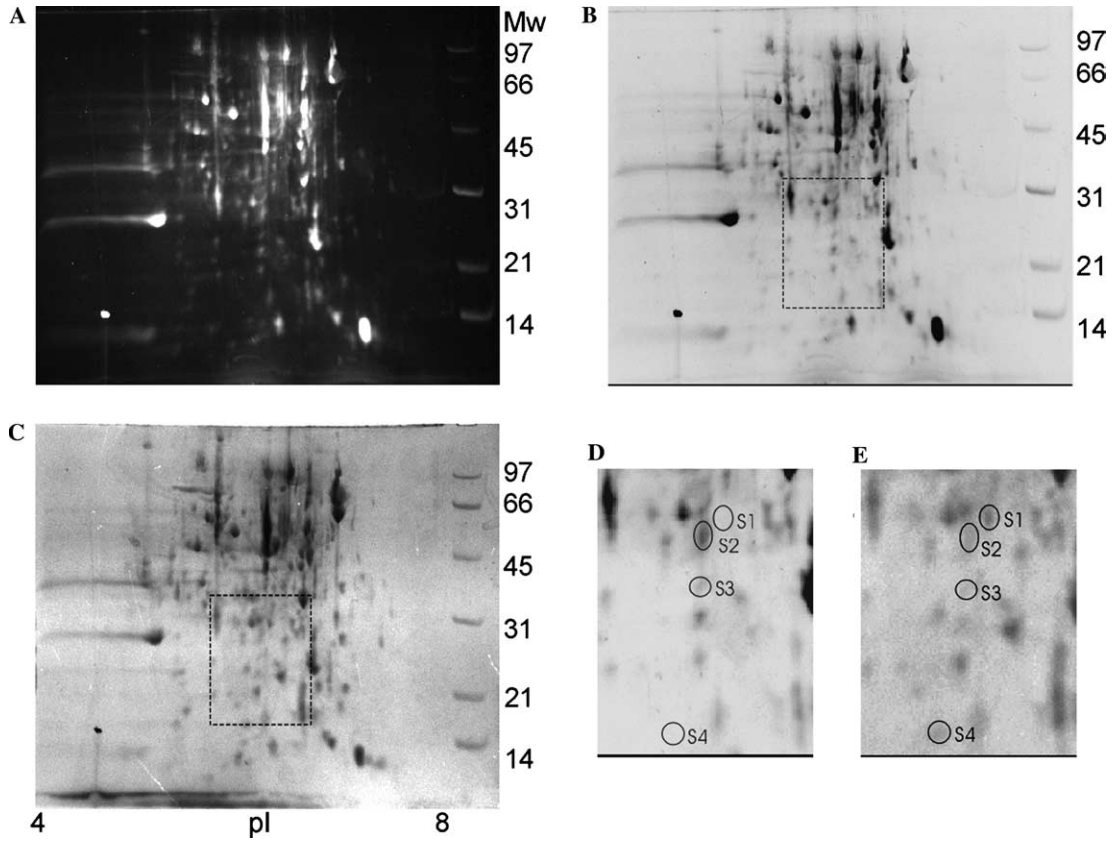

Fig. 6. TCE in-gel visualization can be used for two-dimensional gel electrophoresis. (A) TCE in-gel visualization of 250 μg of *E. coli* extract on a 12% SDS–PAGE gel. (B) Inverted picture of A. (C) CBB stain of the same gel shown in A. (D) Blowup of area highlighted in B. (E) Blowup of area highlighted in C.

membrane-spanning regions of integral membrane proteins have a higher percentage of tryptophans than globular proteins [18,19]. Furthermore experience in our lab indicates that they do not stain as well with CBB. Zhong et al. [12] have already taken advantage of staining membrane proteins with trihalocompounds.

TCE in-gel visualization allows nearly immediate protein detection in PAGE. The ability to use TCE in-gel visualization with 2-DE suggests that this technique will lend itself to automated and high-throughput technologies for proteomics. Furthermore tryptophan visualization will complement other staining techniques to allow detection of proteins not stained efficiently by these methods.

Acknowledgments

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