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Previsible silver staining of protein in electrophoresis gels with mass spectrometry compatibility

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ABSTRACT

A convenient silver staining method for protein in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gels is described. The method is previsible, sensitive, and mass spectrometry (MS) compatible. Two visible counter ion dyes, ethyl violet (EV) and zincon (ZC), were used in the first staining solution with a detection limit of 2 to 8 ng/band in approximately 1 h. The dye-stained gel can be further stained by silver staining, which is based on acidic silver staining employing ZC with sodium thiosulfate as silver ion sensitizers. Especially, ZC has silver ion reducing power by cleavage of the diazo bond of the dye during silver reduction. The second silver staining can be completed in approximately 1 h with a detection limit of 0.2 ng/band.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)[2] is a reliable and widely used analytical technique for the separation and identification of proteins in life sciences. A staining method compatible with the two-dimensional electrophoresis (2-DE) technique and downstream mass spectrometry (MS) identification is crucial for proteomic research. Especially, with the rapid development of proteomics, MS-compatible highly sensitive protein detection methods are becoming more important [1–3]. Various protein detection methods, including organic dye, silver, fluorescence, and radiolabeling, have been developed. Of these methods, organic dye and silver staining are used conventionally because special instruments are not needed. Organic dye staining methods are reproducible, low cost, and compatible with MS but have low sensitivities (10–100 ng/band) [3,4]. In general, silver staining methods have relatively high sensitivity but need multiple steps and lack reproducibility [5–9]. In addition, some silver ion sensitizers used in silver staining may interfere with protein identification due to the introduction of cross-links in proteins [10,11]. Recently, many fluorescent staining methods have been developed, including the SYPRO family and Deep Purple [12–15]. Fluorescent staining methods (0.5–10 ng/band) are more sensitive than dye-based methods, with better linear dynamic range, reproducibility, and MS compatibility; however, for data reading of fluorescent-stained gels, special handling processes and instruments are inevitably needed. Furthermore, most fluorescent staining methods are not suitable for routine use in many laboratories due to their cost. As a result, only a few staining protocols are applied in routine research considering their potential speed, sensitivity, MS compatibility, and cost.

In the current study, to facilitate proteomic research, a combined dye and silver staining method, namely EZ–silver (EZ: ethyl violet [EV] and zincon [ZC]), was developed. Dye staining is an independent staining procedure that was developed by our group in 2004. The detection limit is 2 to 8 ng/band in approximately 1 h. Subsequently, dye-stained gels can be restained by silver staining, which is based on the acidic silver staining method applying ZC and sodium thiosulfate as silver ion sensitizers. Especially, ZC has silver ion reducing power by cleavage of the diazo bond of the dye during silver reduction. The silver staining can be completed in approximately 1 h with a detection limit of 0.2 ng/band after dye staining. This combined staining method has some advantages over other methods. First, because it is a previsible silver staining method before the silver staining, one can decide whether silver staining is necessary or not. Second, both dye staining and silver staining are compatible with MS; therefore, interesting protein spots can be selected from different staining stages for

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[2] *Abbreviations used:* SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; 2-DE, two-dimensional electrophoresis; MS, mass spectrometry; EZ, ethyl violet and zincon; EV, ethyl violet; ZC, zincon; APS, ammonium persulfate; TEMED, tetramethylethylenediamine; APS, ammonium persulfate; CBBR, Coomassie Brilliant Blue R; CBBG, Coomassie Brilliant Blue G; GA, glutaraldehyde; BSA, bovine serum albumin; OVA, ovalbumin; CA, carbonic anhydrase; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; IPG, immobilized pH gradient; MeOH, methanol; EtOH, ethanol; HAc, acetic acid; DW, deionized water; 1-DE, one-dimensional electrophoresis; IEF, isoelectric focusing; TCA, trichloroacetic acid; ACN, acetonitrile; MALDI, matrix-assisted laser desorption/ionization; TOF, time-of-flight; ACTH, adrenocorticotropic hormone; NCBI, National Center for Biotechnology Information.

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protein identification. Third, the newly presented staining method is rapid, sensitive, and low cost.

Materials and methods

Materials

Acrylamide, Bis, tetramethylethylenediamine (TEMED), ammonium persulfate (APS), Tris base, glycine, SDS, EV, ZC, iodoacetamide, glycerol, bromophenol blue, silver nitrate, Coomassie Brilliant Blue R (CBBR), Coomassie Brilliant Blue G (CBBG), glutaraldehyde (GA), sodium thiosulfate, potassium ferricyanide, formaldehyde, trypsin, potassium carbonate, and molecular weight marker proteins (SDS-6H), including myosin heavy chain, β-galactosidase, phosphorylase *b*, bovine serum albumin (BSA), ovalbumin (OVA), and carbonic anhydrase (CA), were purchased from Sigma–Aldrich (St. Louis, MO, USA). Ethylenediaminetetraacetic acid (EDTA), Chaps, dithiothreitol (DTT), phenylmethylsulfonyl fluoride (PMSF), urea, immobilized pH gradient (IPG) strip, cover oil, and IPG buffer were obtained from GE Healthcare (Piscataway, NJ, USA). All other chemicals used were of analytical grade and obtained from various commercial sources.

Solution preparation

EZ staining solution was prepared to be 0.004% ZC/0.003% EV by diluting the stock dye solutions of 0.4% ZC [in methanol (MeOH)] and 0.3% EV [in ethanol (EtOH)] with 24% EtOH/7% acetic acid (HAc). Silver nitrate stock solution (25%) was prepared with deionized water (DW) and filtered twice. Silver staining solution was prepared by the addition of 1 ml of silver nitrate stock solution to 99 ml of DW followed by 80 μl of commercial formaldehyde, whereas the developing solution was initially prepared as two stock solutions consisting of 30% potassium carbonate (solution A) and 0.04% sodium thiosulfate (solution B), both dissolved in DW. Working solution was prepared by mixing the two stocks to result in a final concentration of 3% potassium carbonate and 0.0004% sodium thiosulfate, followed by the addition of 60 μl of commercial formaldehyde in every 100 ml of solution. Using the aforementioned procedure, up to 1 l of staining solution can be easily prepared for approximately 10 pieces of one-dimensional SDS–PAGE gels (e.g., 10 × 8 × 0.1 cm) simultaneously.

Working solutions should be freshly prepared for each reaction. The stock solutions, on the other hand, are stable for several months when stored in tightly sealed and foil-wrapped glass bottles at room temperature.

Gel electrophoresis

Preparation and separation of protein sample in 1-D SDS–PAGE

For one-dimensional electrophoresis (1-DE), *Escherichia coli* BL21 cells were chosen. Proteins were extracted and solubilized from *E. coli* BL21 cells using the following method. *E. coli* cells were harvested by centrifugation at 3000 rpm for 10 min, sonicated in buffer containing 50 mM Tris (pH 7.5), 1 mM EDTA, and 0.4 mM PMSF five times for 1 min, and centrifuged at 15,000 rpm for 20 min at 4 °C. The protein amount in the supernatant was determined by the Bradford method using the Bio-Rad protein assay kit (Hercules, CA, USA) [16]. Molecular weight marker proteins (SDS-6H) and *E. coli* extract were dissolved in buffer containing 60 mM Tris (pH 6.8), 25% glycerol, 2% SDS, 1% DTT, and 0.1% bromophenol blue for 1-DE. Prior to electrophoresis, protein samples were heated at 100 °C for 5 min in a boiling water bath and then cooled to room temperature. Twofold serial dilutions of marker proteins and *E. coli* samples were loaded onto gels at loadings of 0.2 to 100 ng/band and 2 to 1000 ng/well, respectively.

Electrophoresis was carried out on polyacrylamide slab gels (60 × 80 × 0.75 mm) using the discontinuous buffer system of Laemmli [17]. The 4.5% stacking gel was overlaid on the separating gel of 10% polyacrylamide with an acrylamide/Bis ratio of 30:0.8. The running buffer consisted of 0.025 M Tris, 0.2 M glycine, and 0.1% SDS. The gels were run in a Mini-Protein III dual slab cell (Bio-Rad) at a constant current of 22 mA per slab gel using a Power PAC 300 (Bio-Rad).

Preparation and separation of protein sample in two-dimensional SDS–PAGE

For 2-D electrophoresis, the total cell proteins of *E. coli* were separated using IPG gel strips (13 cm, linear 4–7 pH gradient). The samples mixed with rehydration buffer (8 M urea, 2% Chaps, 2% IPG buffer, 0.04 M DTT, 1× nuclease solution, and a few grains of bromophenol blue) were rehydrated in an Immobiline DryStrip Reswelling Tray (GE Healthcare). Isoelectric focusing (IEF) was performed using a Multiphor II electrophoresis unit (GE Healthcare) according to the manufacturer's instructions. IPG strips containing the samples were transferred to the focusing tray and covered with mineral oil. IEF was carried out using a five-step program (0–150 V for 1 h with gradient, 150–300 V for 3 h, 300–1500 V for 1.5 h, 1500–3500 V for 1.5 h, and hold at 3500 V for 5 h). On completion of the first-dimensional electrophoresis, the IPG gel strips were removed from the strip holder. The excess mineral oil was allowed to drip from the IPG strip. Subsequently, strips were incubated in 10 ml of the first equilibration buffer (1% DTT, 50 mM Tris–HCl [pH 8.8], 6 M urea, 30% glycerol, 2% SDS, and 0.1% bromophenol blue) for 15 min and in 10 ml of the second equilibration buffer (2.5% iodoacetamide, 50 mM Tris–HCl [pH 8.8], 6 M urea, 30% glycerol, 2% SDS, and 0.1% bromophenol blue) for 15 min prior to the second-dimensional electrophoresis. The IPG strips should be placed in individual tubes with the support film facing toward the tube wall, and the tubes were shaken on a rocker. To transfer the IPG gel strips for the second dimension, the separating gel of 11.5% polyacrylamide with an acrylamide/Bis ratio of 30:0.8 was cast using a Hoefer SE 600 system (Amersham Biosciences, Piscataway, NJ, USA). IPG strips were then sealed on top of SDS–PAGE gels with agarose and were run at a constant current of 22 mA per slab gel using power PAC 300 (Bio-Rad) with a running buffer consisting of 0.025 M Tris, 0.2 M glycine, and 0.1% SDS.

Protein staining

All working solutions should be prepared just before use with DW and clean glassware or plasticware. All steps were carried out at room temperature with shaking.

CBBR–silver staining method

This staining method was essentially according to Irie and coworkers [18]. For staining with CBBR–silver, proteins were fixed and stained at the same time by immersing the gels for 1 h in 5% formaldehyde/25% EtOH/10% HAc and then in 0.5% formaldehyde/25% EtOH/10% HAc for 3 h in the presence of 0.05% CBBR. For further silver staining, gels were destained in 25% isopropanol/10% HAc followed by 10% isopropanol/10% HAc with repeated changes until the background became clear. Then gels were washed three times with 10% EtOH for 5 min each, 30 min in 1.43% sodium cacodylate buffer solution, and four times incubation in 10% MeOH for 5 min each. After that, gels were stained with cupric nitrate/silver nitrate solution for 30 min, followed by 10 min incubation in fresh ammoniacal silver (diamine) solution. Finally, gels were developed with a reducing solution containing 0.025% formaldehyde, 0.006% citric

acid, and 10% EtOH until the desired protein bands was attained. The silver development could be stopped by immersing the gel into 1% HAc.

CBBG–silver staining method

This staining method was essentially according to De Moreno and coworkers [19]. Electrophoretic gels were fixed by three changes of 200 ml of 40% MeOH/10% HAc solution for 20 min each and stained in 100 ml of CBBG staining solution (0.25% CBBG in 50% MeOH/12.5% trichloroacetic acid [TCA]) for 30 min. Then the gels were destained by three changes of 100 ml of 5% TCA for 10 min each. For further silver staining, the CBBG-stained gels were washed two times for 10 min in 40% MeOH/10% HAc, followed by two times washing in 10% EtOH/5% HAc for 5 min each. Then gels were soaked for 10 min in 100 ml of 3.4 mM potassium dichromate/0.032 N nitric acid, followed by three times washing with DW for 10 min each. After that, gels were stained with 100 ml of 12 mM silver nitrate for 30 min and washed with DW for 0.5 min. Then silver-stained gels were reduced by soaking in 0.28 M sodium carbonate/0.5 ml of formaldehyde per liter for 3 to 5 min. Finally, the staining was stopped by 5 min incubation in 5% HAc.

EZ–silver staining method

The dye and silver staining procedures are summarized in Table 1. A modified EZ–dye staining protocol was used as follows [20,21]. After electrophoresis, gels were fixed in 200 ml of fixing solution (40% EtOH/10% HAc solution) for 30 min (or overnight). Gels were then stained in 100 ml of 0.0034% ZC/0.003% EV/24% EtOH/7% HAc for 30 to 60 min (or for several days). Gels can be dried or further stained by silver staining. For gel drying, the stained gels should be washed in 100 ml of fixing solution for 30 s followed by 100 ml of DW for 5 min to remove dyes on the gel surface and also to improve band intensity. Then gels were dried on a filter paper under vacuum at 65 °C for 40 min or used for MS analysis. For further silver staining, the dye-stained gels were performed by the EZ–silver staining protocol below.

After EZ–dye staining, gels were destained in 200 ml of fixing solution for 20 min and sensitized with 100 ml of 0.02% (w/v) sodium thiosulfate in fixing solution for 10 min, followed by washing in 200 ml of DW for 3 × 5 min. After that, gels were impregnated in 100 ml of 0.25% (w/v) silver nitrate containing 0.03% (w/v) formaldehyde for 10 min, washed in 200 ml of DW for 2 × 20 s, and immersed in 100 ml of 3% (w/v) potassium carbonate, 0.022% formaldehyde, and 0.0004% (w/v) sodium thiosulfate solution for 5 to 8 min to develop image. After silver ion reduction, gels were immersed in 100 ml of 1.5% (w/v) EDTA for 10 min (or overnight) to stop the development. Finally, stained gels were dried on filter paper under vacuum at 65 °C for 40 min or used for MS analysis. The volumes of the working solutions used in each step were proportional to the gel size, for example, 400 ml of fixing solution for a 15 × 15-cm (2-D) regular gel (as shown in Table 1).

GA–silver staining method

Silver nitrate staining using GA as a sensitizer was performed using a modified method described by Heukeshoven and Dernick [8]. Briefly, after electrophoresis, gels were fixed in 125 ml of 40% (v/v) EtOH and 10% (v/v) HAc solution for 30 min, then reacted in 125 ml of 6.8% sodium acetate, 0.125% GA, and 0.2% sodium thiosulfate solution for 30 min, and finally washed in 125 ml of DW for 3 × 5 min. Gels were then impregnated in 125 ml of 0.015% formaldehyde and 0.25% silver nitrate solution for 20 min, washed in 125 ml of DW for 2 × 1 min, and immersed in 125 ml of 3% sodium carbonate and 0.007% formaldehyde solution. After silver ion reduction, gels were immersed in 125 ml of 1.5% EDTA for 10 min to stop development. Stained gels were dried on a filter paper under vacuum at 65 °C for 40 min. The volumes of the working solutions used in each step were proportional to the gel size. Twofold volume of working solutions discussed above was applied for further 2-D gel stain.

Image analysis

After gel drying, the quantization of the protein bands was performed with a scanner (SIS 3800, Samsung, South Korea) interfaced with a Samsung computer (DV 25, Samsung). Image analysis was performed by using the TINA 2.09 software program (Raytest, Straubenhardt, Germany).

MALDI–TOF MS analysis

After detecting molecular weight marker proteins (SDS-6H) in 1-D gels with EZ–silver, proteins were subjected to trypsin digestion and MS. In-gel digestion was performed in three steps following the protocol modified by Russell and coworkers [22].

The gel pieces were destained with mixing solution of fresh 100 mM sodium thiosulfate and 30 mM potassium ferricyanide with a ratio of 1:1 for 10 min, and the gel pieces were washed twice for 15 min with 1 ml of DW. Then the gel slices were washed with DW and 50% acetonitrile (ACN) and incubated with 100% ACN for 10 min. Opaque gels were equilibrated with 100 mM ammonium bicarbonate (pH 8.0) for 5 min, and then an equal volume of 100% ACN was added for 15 min. After removing the solution, the dried gel pieces were reduced with 10 mM DTT containing 100 mM ammonium bicarbonate at 56 °C for 45 min and alkylated with 55 mM iodoacetamide containing 100 mM ammonium bicarbonate (prepared freshly just before use) for 30 min in the dark. After removing the solution,

Table 1
EZ–silver staining protocol of protein in SDS–PAGE (0.75 or 1.0 mm, 8 × 10 cm [1-D gel] or 15 × 15 cm [2-D gel])

Stain	Steps	Reagents	Volume		Time
			1-D (ml)	2-D (ml)	
EZ	Fix	40% (v/v) EtOH/10% (v/v) HAc	200	400	30 min (or overnight)
	Stain	0.0034% (w/v) ZC, 0.003% (w/v) EV, 24% (v/v) EtOH/7% (v/v) HAc	100	200	30 min (or several hours)
Silver	Destain	40% (v/v) EtOH/10% (v/v) HAc	200	400	20 min
	Enhance	0.02% (w/v) sodium thiosulfate, 40% (v/v) EtOH/10% (v/v) HAc	100	200	10 min
	Rinse	Deionized water	200	400	3 × 5 min
	Silver	0.25% (w/v) silver nitrate, 0.03% (w/v) formaldehyde	100	200	10 min
	Rinse	Deionized water	200	400	2 × 20 s
	Develop	3% (w/v) potassium carbonate, 0.022% (w/v) formaldehyde, 0.0004% (w/v) sodium thiosulfate	100	200	5–8 min
	Stop	1.5% (w/v) EDTA	100	200	10 min (or overnight)

the gels were washed with 100 mM ammonium bicarbonate, and an equal volume of 100% ACN was added and incubated. The gel pieces were dried in a SpeedVac for 15 min. Finally, the gel pieces were digested with trypsin solution at 4 °C for 45 min, and the resulting peptide mixtures were analyzed by matrix-assisted laser desorption/ionization (MALDI)–MS. To remove salt or contaminants from the peptide mixture, it was purified with ZipTip$_{C18}$ (Millipore, Bedford, MA, USA). MS analysis was performed on an Applied Biosystems Voyager System 4239 MS (PerSeptive Biosystems, Framingham, MA, USA). The parameters of MS analysis were as follows: mode of operation, reflector; extraction mode, delayed; polarity, positive; accelerating voltage, 20,000 V; grid voltage, 63%; mirror voltage ratio, 1.12; extraction delay time, 100 ns; acquisition mass range, 850 to 3500 Da; number of laser shots, 150/spectrum; laser intensity, 1445; laser rep rate, 20.0 Hz; calibration type, external–new standard; calibration matrix, α-cyano-4-hydroxycinnamic acid; low mass gate, 500 Da; timed ion selector, off; bin size, 0.5 ns; vertical scale 0, 500 mV; vertical offset, 0.75%; input band with 0, 500 MHz; instrument name, Voyager-DE STR; shots in spectrum, 150; TIS gate width, 15; TIS flight length, 1184. MALDI–time-of-flight (TOF) MS spectra were processed by Data Explorer software (version 4.0, Applied Biosystems, Foster City, CA, USA), and m/z peak evaluation was performed by MoverZ software (ProteoMetrics, New York, NY, USA). Masses were internally calibrated using standard peptides: angiotensin, $[M+H]^+ = 1296.6853$; adrenocorticotropic hormone (ACTH) 18–39, $[M+H]^+ = 2465.1989$. Protein identification was performed by searching in the National Center for Biotechnology Information (NCBI 20070601) protein database using Mascot software (version 2.2, Matrix Science, London, UK). The search was based on three assumptions: (i) that the polypeptides are monoisotopic, (ii) that the peptides may be oxidized at methionine residues, and (iii) that the peptides are carbamidomethylated at cysteine residues. Also, one missed trypsin cleavage and a mass tolerance of 50 ppm were allowed for matching the peptide mass values. For positive identification, the score of the result of $[-10 \times \log(P)]$ needed to exceed the significance threshold level ($P < 0.05$).

Results

Protein detection in 1-D and 2-D SDS–PAGE

To compare the sensitivities of the EZ–dye, EZ–silver, and GA–silver staining methods, marker proteins (SDS-6H) were sep-

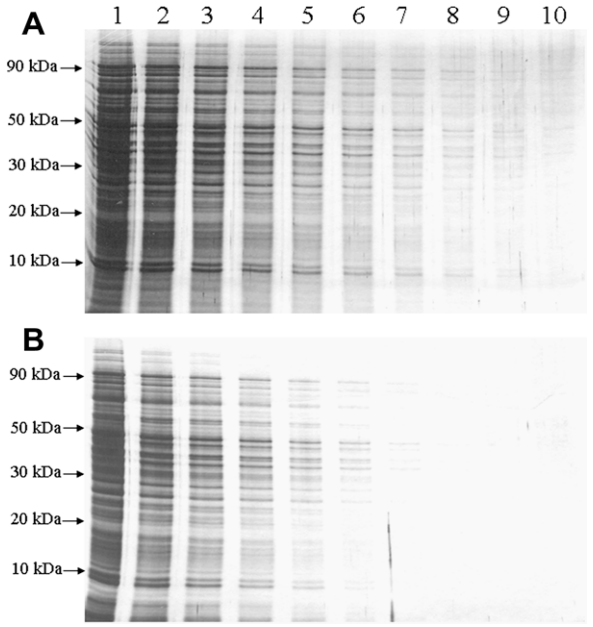

Fig. 2. Comparison of the sensitivities of EZ–silver and GA–silver stains in 1-D SDS–PAGE with total cell proteins of *E. coli* BL21. Each of staining procedures was performed as described in Materials and methods: (A) EZ–silver stain; (B) GA–silver stain. Twofold serial dilutions of protein loaded onto the gel were as follows: lane 1, 1000 ng; lane 2, 500 ng; lane 3, 250 ng; lane 4, 125 ng; lane 5, 62.5 ng; lane 6, 31.3 ng; lane 7, 16 ng; lane 8, 8 ng; lane 9, 4 ng; lane 10, 2 ng. For EZ–silver stain, gels were stained in 100 ml of EZ staining solution for 30 min after 30 min fixing.

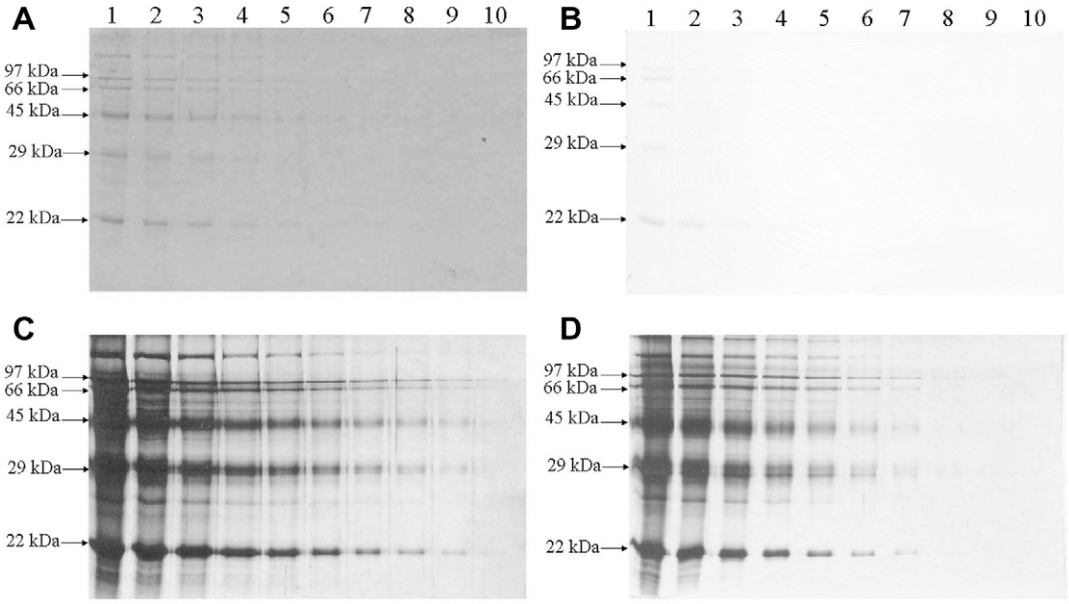

Fig. 1. Comparison of the sensitivities of EZ–dye, EZ–silver, and GA–silver stains in 1-D SDS–PAGE with standard marker proteins. Each of staining procedures was performed as described in Materials and methods: (A) EZ–dye; (B) destained gel for further silver stain; (C) EZ–silver; (D) GA–silver. Twofold serial dilutions of SDS-6H marker proteins loaded onto the gel were as follows: lane 1, 100 ng/band; lane 2, 50 ng/band; lane 3, 25 ng/band; lane 4, 12.5 ng/band; lane 5, 6.3 ng/band; lane 6, 3.2 ng/band; lane 7, 1.6 ng/band; lane 8, 0.8 ng/band; lane 9, 0.4 ng/band; lane 10, 0.2 ng/band. For EZ–silver stain, gels were stained in 100 ml of EZ staining solution for 30 min after 30 min fixing.

arated and stained by three different methods (Fig. 1). The detection limits of the EZ–dye and EZ–silver methods were 2 to 8 ng/band and 0.2 ng/band, respectively. The sensitivities of the EZ–silver method were two- to fourfold higher than those of the GA–silver method. To further confirm sensitivities and to compare protein staining patterns, total *E. coli* cell proteins were separated by 1-DE and 2-DE and were stained by both silver staining methods. 1-DE-stained patterns are shown in Fig. 2. EZ–silver staining was found to present a better resolution than GA–silver. 2-DE-stained patterns are shown in Fig. 3. The 2-DE protein map of the EZ–silver method showed more spots than that of the GA–silver method with better contrast. Moreover, there were no negative spots in the EZ–silver patterns, in contrast to the GA–silver patterns. The differential staining of proteins is due primarily to the different mechanisms by which each stain reacts with protein. For GA–silver, GA acting as a sensitizing agent can form cross-linking with amine groups to increase the speed of silver reduction [23,24], whereas EZ–silver has been shown to be greatly influenced by the diazoic group in ZC that has silver ion reducing power by cleavage of the diazo bond of the dye [25–28]. In conclusion, the EZ–silver method has better sensitivity and resolution than the GA–silver method.

EZ–silver staining was also compared with results obtained from CBBG–silver and CBBR–silver with respect to staining time and detection limit for low-nanogram amounts of protein. As shown in Fig. 4, the sensitivity achieved when using EZ–silver stain to visualize SDS–PAGE was generally similar to that achieved with the CBBR–silver staining technique, which can detect approximately 0.2 ng of the same marker protein, whereas the sensitivity of the CBBG–silver staining technique was 1.6 ng, approximately eightfold lower compared with that of EZ–silver staining. Moreover, to achieve highly sensitive staining of proteins in SDS–PAGE, unwanted background staining was obtained by both CBBR–silver and CBBG–silver stains, and this can greatly affect the contrast between the protein bands and background. In addition, high-concentration formaldehyde fixation of CBBR–silver staining could increase the staining intensity of proteins; however, it also interfered with the compatibility of this method with peptide MS through the formation of cross-linking between amino acid reactive side chains and formaldehyde [10,11,29]. On the other hand, to complete the protocol, staining was carried out for 3.8 h for CBBG–silver staining and for 7 h for CBBR–silver staining, as compared with a 2-h protocol for EZ–silver staining, similar to GA–silver staining.

Compatibility with MALDI–TOF MS

To determine the compatibility of the EZ–silver method with MALDI–TOF MS, in-gel trypsin digestion of protein bands was performed on marker proteins stained with EZ–silver. MALDI–TOF MS spectra were acquired from each of the proteins bands and evaluated: β-galactosidase (*E. coli*, 116 kDa, p*I* 5.28), phosphorylase *b* (rabbit, 97 kDa, p*I* 6.77), BSA (bovine, 71 kDa, p*I* 5.82), OVA (chick, 43 kDa, p*I* 5.20), and CA (bovine, 29 kDa, p*I* 6.41). The summarized MALDI–TOF MS data of these five proteins at loadings from 3 to 200 ng are provided in Table 2. (Mascot scores of 6 and 3 ng BSA did not exceed the significance threshold; thus, they cannot be seen as positive identification.)

Linear dynamic range of EZ–silver staining method

In terms of the linear dynamic range of EZ–silver staining, stained density and band areas were determined by using the scanned data from standard proteins. The linear dynamic ranges (and correlation coefficients) of the amounts of proteins were as follows: myosin, 0.4 to 50 ng (correlation coefficient 0.937); phosphorylase *b*, 0.4 to 50 ng (0.904); BSA, 0.4 to 50 ng (0.913); OVA, 0.4 to 50 ng (0.937); CA, 0.4 to 50 ng (0.937).

Discussion

In this study, a new protein silver staining method, namely EZ–silver, was developed. In EZ–dye staining, its possible staining mechanism is similar to that of calconcarboxylic acid or CBB-based dye staining. Moreover, the diazoic functional group in ZC has silver ion reduction power in alkaline solution that can be incorpo-

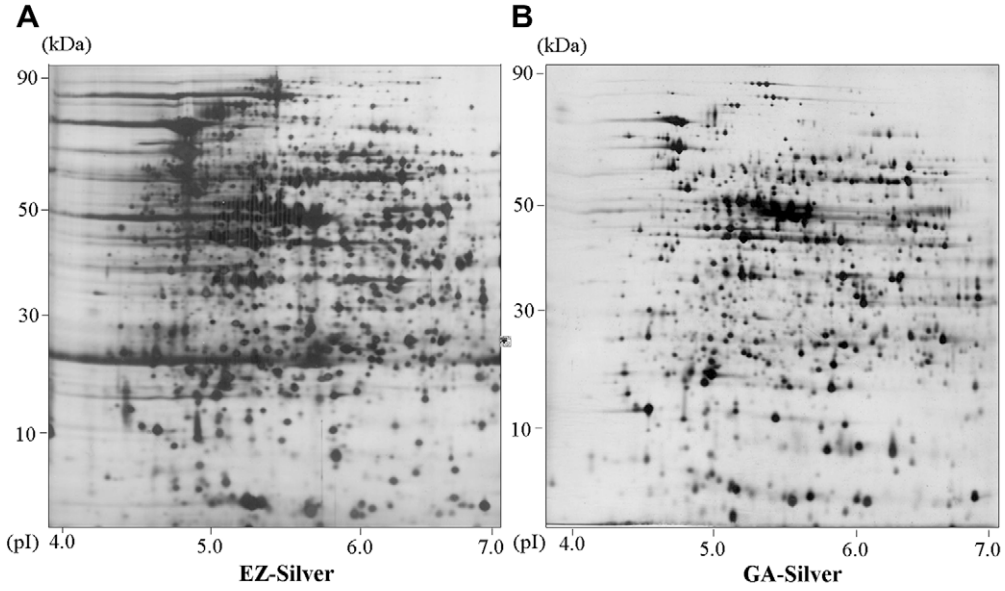

Fig. 3. Comparison of the sensitivities of EZ–silver and GA–silver stains in 2-D SDS–PAGE with total cell proteins of *E. coli* BL21. Each of staining procedures was performed as described in Materials and methods: (A) EZ–silver stain; (B) GA–silver stain. Here 100 μg of *E. coli* BL21 total cell protein sample per gel was loaded in linear IPG strips (13 cm, pH 4–7). For EZ–silver stain, gels were stained in 200 ml of EZ staining solution for 30 min after 30 min fixing.

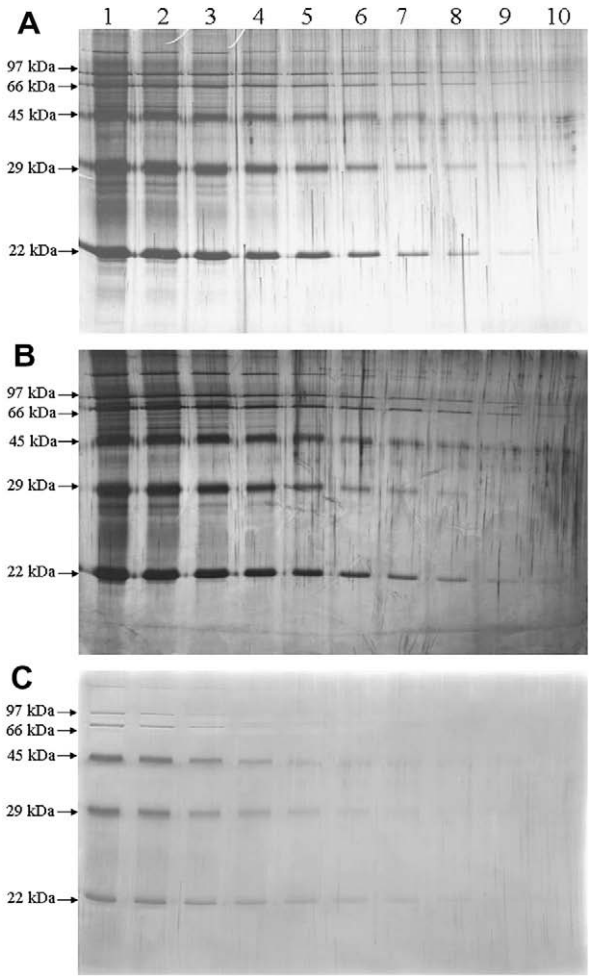

Fig. 4. Comparison of the sensitivities of EZ–silver, CBBG–silver stain, and CBBR–silver stain in 1-D SDS–PAGE with standard marker proteins. Each of staining procedures was performed as described in Materials and methods: (A) EZ–silver stain; (B) CBBR–silver stain; (C) CBBG–silver stain. Twofold serial dilutions of protein loaded onto the gel were as follows: lane 1, 1000 ng; lane 2, 500 ng; lane 3, 250 ng; lane 4, 125 ng; lane 5, 62.5 ng; lane 6, 31.3 ng; lane 7, 16 ng; lane 8, 8 ng; lane 9, 4 ng; lane 10, 2 ng. For EZ–silver stain, gels were stained in 100 ml of EZ staining solution for 30 min after 30 min fixing.

rated with formaldehyde in the course of silver nucleation and reduction. Previous work indicates that the optimal fixing and staining times were 30 min and 30 to 60 min, respectively. According to the results, as little as 4 to 8 ng of protein bands can be visualized in approximately 30 to 60 min staining, and more than 60 min staining resulted in a detection limit plateau [25–28]. Therefore, prolonged staining does not affect the intensity of protein staining, making its use redundant. To enhance sensitivity and minimize staining time, 30 min fixing followed by 30 min staining was applied in this study, as shown in Table 1. Moreover, for further silver staining, the prolonged staining time also does not interfere with the destaining step; a clear background can be achieved with 30 min destaining in 40% EtOH/10% HAc, as shown in Fig. 1B.

Also, in many silver staining protocols, gel buffer will cause low sensitivity and point streaking. Therefore, degassing of gel buffer before polymerization is strongly recommended. Furthermore, the reductant used during sample preparation should be no more than 2% of 2-mercaptoethanol or 1% of DTT to avoid point streaking in silver staining. For 2-DE, it is necessary to scavenge any excess thiol reducing agent with iodoacetamide or to use thiol-free reducing agent before loading an IEF strip on 2-DE.

In addition, the detection methods required in proteomic research should be compatible with MS analysis. However, many staining procedures can cause modifications of protein. For example, methylation (or ethylation) takes place preferentially at glutamic acid residues due to either TCA or MeOH (or EtOH) in staining solutions, alkylation of cysteine residues takes place in the equilibration step of 2-DE during iodoacetamide treatment, and lysine can form protein cross-links in the presence of GA or formaldehyde [30,31]. One should be aware that silver staining could inherently modify proteins; it is only different in level. For EZ–silver staining, two independent staining procedures were composed in this method. First, the dye staining step has no direct covalent bonding between ZC and protein; thus, it can be fully compatible with MS as described in previous work. Second, with EZ–silver staining, formaldehyde has a potential risk of modifying proteins, but the amount of formaldehyde used is very small and ZC plus thiosulfate as silver ion sensitizers are also compatible with MS [28]. Therefore, EZ–silver staining can be compatible with MS analysis.

Table 2
MALDI–TOF MS data for five standard proteins with EZ–silver-stained gels

Protein ID		200 ng/band	100 ng/band	50 ng/band	25 ng/band	12 ng/band	6 ng/band	3 ng/band
β-Galactosidase	Mascot sore	187	213	188	73	127	117	62
	Expect value	1.1e-14	2.7e-17	8.7e-15	0.0025	1.1e-08	1.1e-07	0.034
	Peptide matched	23	27	20	14	22	20	11
	Sequence coverage	33%	32%	30%	18%	25%	24%	14%
Phosphorylase *b*	Mascot sore	270	146	165	78	128	139	86
	Expect value	5.8e-22	1.4e-09	1.8e-11	0.0081	9.1e-08	7.3e-09	0.0019
	Peptide matched	29	29	27	16	25	23	15
	Sequence coverage	40%	39%	37%	26%	38%	34%	24%
BSA	Mascot sore	99	111	89	79	114	67	66
	Expect value	7.6e-05	4.6e-06	0.00066	0.0078	2.3e-06	0.11	0.19
	Peptide matched	12	14	12	9	15	10	9
	Sequence coverage	23%	28%	22%	14%	27%	19%	16%
OVA	Mascot sore	87	87	80	77	71	80	–
	Expect value	0.0016	0.0017	0.0079	0.016	0.067	0.0079	–
	Peptide matched	11	9	9	7	8	8	–
	Sequence coverage	35%	33%	29%	27%	30%	30%	–
CA	Mascot sore	90	97	79	80	81	98	–
	Expect value	0.00063	0.001	0.0076	0.0056	0.0049	8.9e-05	–
	Peptide matched	11	10	9	11	8	8	–
	Sequence coverage	60%	53%	48%	57%	55%	50%	–

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