Xu Wang[1]
Sun-Young Hwang[1]
Wei-Tao Cong[2]
Li-Tai Jin[2]
Jung-Kap Choi[1]

[1]College of Pharmacy and Research Institute of Drug Development, Chonnam National University, Gwangju, South Korea
[2]School of Pharmacy, Wenzhou Medical College, Wenzhou, Zhejiang, P. R. China

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Research Article

Promoted negative staining of proteins in SDS-PAGE using Eosin B compounded with magnesium chloride

In this study, we describe an effective visualizing technique for proteins in SDS-PAGE based on the organic dye, Eosin B, the sensitivity of which can be further strengthened by the addition of magnesium to the staining solution after electrophoresis. The newly developed protocol is low in cost and easily performed compared with the common methods for protein analysis in 1-D and 2-D gels. It provides a much better sensitivity (0.2 ng of single protein band) than that of imidazole–zinc negative stain for fixing and staining within 1 h, and an excellent performance in terms of compatibility with MALDI-TOF MS. The results show that similar identification scores and numbers of matched peptides were obtained by both methods. Furthermore, the effects of different metal salts on the quality of protein visualization by Eosin B were also investigated. Because of its sensitivity, stability, and safety, this stain may be a more practical method for protein determination in the routine laboratory.

Keywords:
Eosin B / Magnesium chloride / Negative stain / PMF / Proteomics
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1 Introduction

With the development of proteomics, the visualizing of gel-separated proteins has become an important strategic means for research of proteins on mono- or bi-dimensional gels. Most existing common methods available for staining of proteins in SDS-PAGE are CBBR, silver, SYPRO Ruby and imidazole–zinc (IZ) negative stains. Detection with CBBR is simple, rather linear and compatible with MS techniques, but has long staining/destaining times and low senstitivity [1–4]. The sensitivity of silver stain is far superior to that of CBBR stain, which provides detection of low nanogram amounts of protein. The efficiency was seriously limited resulting from the poor linearity, cumbersome procedures and a relatively poor compatibility with MS [5–7]. SYPRO Ruby stain, one of the most commonly used fluorescent detection methods, has a good linear relationship similar to CBBR stain, and a high detection limit (0.5–1 ng of single protein band) almost equivalent to the highest silver procedures available. But the major drawback of the staining is high cost of the reagent compounded with the safety problems that the gel excision is usually carried out on a UV table [8–10]. On the other hand, although the IZ reverse stain is effective in dealing with many of the limitations of prerequisites with normal techniques, its relatively poor contrast between the band/spot and background makes excision difficult to perform [11–14]. Therefore, as an ideal method, it should combine several features in the estimation of proteins in terms of simplicity, sensitivity, broad linearity, economy, and compatibility with MS analysis [15].

In the previous research, a simple, rapid and high-throughput negative detection technique has been developed in our lab. As an improved procedure, we recently made further researches into the negative detection technique by using Eosin B (EB) (4′, 5′dibromo-2′,7′-dinitrofluorescein) with the aiding of metal salts to strengthen the sensitivity [16]. EB is a heterocyclic dye (Fig. 1), which has been widely used as a counterstain for collagen following Mayer's Hemalum or with azure A to stain cells granules, nuclei, microorganisms, and for differential staining of anterior pituitary [17]. According to the reports, it has also been used for the estimation of protein under acidic conditions with the detection limit of 1.2 μg BSA by using spectrophotometer [18]. In this study, EB was selected to negatively detect SDS-PAGE separated proteins which can precipitate in the gel background, while absents from those zones where proteins are located through the formation of a stable water-soluble protein–dye complex [19–23]. Furthermore, the results also indicate that this effect of EB can be

Correspondence: Professor Jung-Kap Choi, Laboratory of Analytical Biochemistry, College of Pharmacy, Chonnam National University, Yongbong-Dong 300, Buk-Ku, Kwangju 500-757, South Korea
E-mail: jkchoi@chonnam.ac.kr
Fax: +8262-530-2911

Abbreviations: DW, deionized water**; EB,** Eosin B**; EY,** Eosin Y**; GA,** glutaraldehyde**; HAc,** acetic acid**; IZ,** imidazole–zinc

Colour Online: See the article online to view Figs. 2 and 6 in colour.

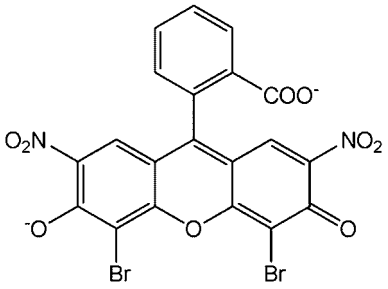

Figure 1. The structure of EB.

greatly improved by the addition of $MgCl_2$ into the staining solution than EB alone. As a result, the contrast and the sensitivity of the staining were improved.

2 Materials and methods

2.1 Materials

Acrylamide, bis, tetramethylenediamine, ammonium persulfate, Tris base, glycine, SDS, iodoacetamide, glycerol, bromophenol blue, ammonium bicarbonate, trypsin, imidazole, zinc sulfate, EB, Eosin Y (EY), SYPRO Ruby gel stain, and protein contained approximately equal amount of mixtures (SDS6H2) of myosin (rabbit muscle, 205 kDa), β-galactosidase (*Escherichia coli*, 116 kDa), phosphorylase b (rabbit muscle, 97 kDa), BSA (bovine, 71 kDa), ovalbumin (chicken egg, 45 kDa) and carbonic anhydrase (bovine erythrocytes, 29 kDa) were obtained from Sigma-Aldrich Chemical (St. Louis, MO, USA). PlusOne silver staining kit was purchased from GE Healthcare. CHAPS, DTT, PMSF, urea, IPG strip, cover oil and IPG buffer were from Amersham Biosciences (Uppsala, Sweden). All other chemicals used were of analytical grade and were obtained from various commercial sources.

2.2 Solution preparation

Stock solutions of 5% w/v EB and 40% w/v $MgCl_2$ were prepared with 45% MeOH. Staining solution was prepared to be 0.25% w/v EB and 0.8% w/v $MgCl_2$ by diluting both of the stock solutions with 45% MeOH. The stock solutions are stable to be used in sealed bottles protected from light for several months at room temperature. Staining solution, on the other hand, should be freshly prepared to yield the working concentration just before used.

2.3 Gel electrophoresis

The preparation and separation of protein sample in 1-D and 2-D SDS-PAGE were performed essentially as previously described utilizing 4.5% stacking gel and 10% separating gel with an acrylamide/Bis ratio of 30:0.8 for 1-D SDS-PAGE [24]. For 2-D gel electrophoresis, approximately 100 μg extracted protein sample was applied *per* gel and performed as described previously [24].

2.4 Protein staining

2.4.1 EB stain with magnesium (EB-Mg)

As shown in Fig. 2, after electrophoresis, gels were fixed in 40% EtOH-10% acetic acid (HAc) fixing solution for 20 min, washed in deionized water (DW) for 3 min twice, and then replaced DW with 45% MeOH solution for 10 min. After that, gels were immersed in the staining solution contained 0.25% EB with 0.8% $MgCl_2$ of 45% MeOH for 15 min. Finally, gels were developed in 2% HAc for 5 min. All the gels should be thoroughly immersed in the corresponding solutions during the staining process (typically 50 mL solution for a minigel (6 cm × 8 cm × 0.75 mm) and 200 mL solution for 2-DE) with continuous and gentle agitation.

2.4.2 EB staining

EB stain was essentially performed according to the procedure from above EB-Mg stain, except that $MgCl_2$ was absented in the staining solution.

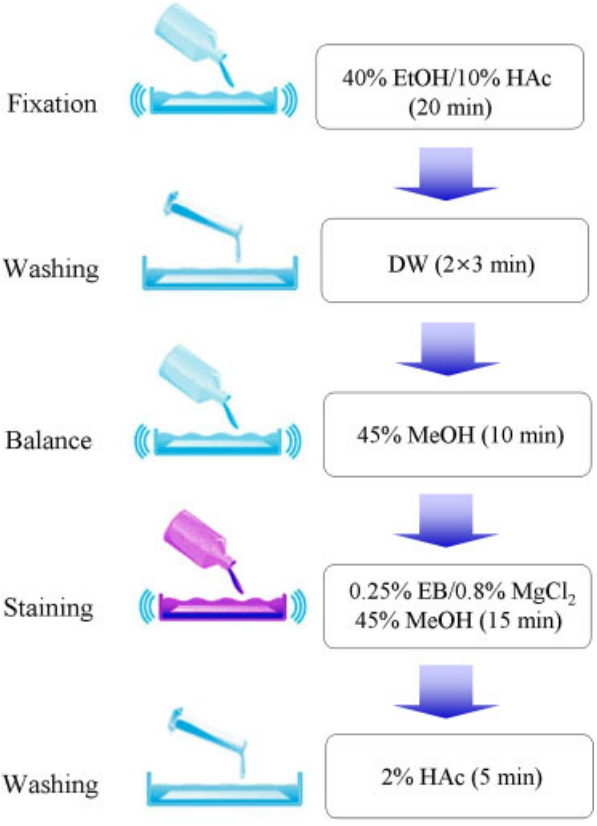

Figure 2. Staining protocol for proteins using EB-Mg.

2.4.3 EY staining

The staining method was essentially performed according to Cong *et al*. [24]. After electrophoresis, gels were fixed in 50% v/v EtOH with 10% v/v HAc solution for 20 min, washed by two changes of DW for 5 min each, followed by 10 min washing in 50% MeOH. Then the gels were immersed in EY staining solution for 15 min. Finally, the gels were developed in DW for 3 min.

2.4.4 IZ staining

The staining method was essentially performed according to Castellanos-Serra *et al*. [14]. After SDS-PAGE, gels were rinsed in DW for 30 s and then incubated in 0.2 M imidazole, 0.1% SDS for 15 min. Once completed, discarded the solution and incubated the gels in 0.2 M zinc sulfate until the gel background becomes a deep white consistency (~30 s), leaving the protein bands transparent and colorless. Staining was stopped by rinsing the gels with abundant DW.

2.4.5 SYPRO Ruby staining

The staining method was essentially performed according to Berggren *et al*. [8]. 2-D gels were fixed after electrophoresis using 10% MeOH, 7% HAc solution for 1 h; fixing was not required for 1-D gel. 1-D gels were placed into 50 mL (300 mL for 2-D gel) SYPRO Ruby staining solution for no less than 3 h. Then the gels were rinsed in 10% MeOH, 7% HAc solution for 30 min. All staining and washing steps were performed with continuous gentle agitation in polypropylene dishes. The containers were wrapped with aluminum foil to avoid light exposure.

2.4.6 Glutaraldehyde (GA)-silver staining

Silver nitrate staining using glutaraldehyde (GA) as a sensitizer was performed using a modified method described by Heukeshoven and Dernick [7]. Briefly, gels were fixed in 125 mL of 40% v/v EtOH, 10% v/v HAc solution for 30 min, and then reacted in 125 mL of 6.8% sodium acetate, 0.125% GA and 0.2% sodium thiosulfate solution for 30 min, and washed in 125 mL DW for 3×5 min. Gels were then impregnated in 125 mL of 0.015% formaldehyde, 0.25% silver nitrate solution for 20 min, washed in 125 mL DW for 2×1 min, and immersed in 125 mL of 3% sodium carbonate, 0.007% formaldehyde solution. After silver ion reduction, gels were immersed in 125 mL of 1.5% EDTA for 10 min to stop development.

2.5 Image analysis and spot-cutting

Gels stained with SYPRO Ruby were scanned at 550 V with the resolution of 200 μm using a Typhoon 9400^{TM} scanner (Amersham Biosciences) with the green laser (532 nm) for excitation and 610 nm band pass emission filter. For EY, EB, EB-Mg and IZ-stained proteins; gels can be visualized both by place the stained gels on a black background or on a daylight lamp view box. Comparing with the gel scanned with a black background, visualized the gel on the daylight lamp view box can provide a little better sensitivity. However, for standard and routine digitalization of the stained gels, gels were only digitized with scanner in this study. Furthermore, during digitalization of the stained gels, all the gels visualized by negative stains of different protocols (EY, EB, EB-Mg, and IZ) were digitized at same adjustment under identical condition for all of the gels to eliminate possible disparity of data values. While, for silver-stained proteins, gels were placed directly on the scanner. All images were adjusted for optimal contrast with resolutions set to 200 dpi to obtain the figures with a scanner (V700, Epson, Seiko, Japan). The images were exported in TIF format and imported for analysis into TINA 2.09. To fulfill the downstream of protein identification by mass spectrometer, gel excision is an inevitable step. For negative-stained gels, however, they face the problem of poor contrast between the protein spots and the background during gel excision, which makes it difficult to perform. But for EB-Mg negative stain, the sensitivity and contrast between the protein spots and background were greatly increased and gel excision of the EB-Mg-stained gels can be easily performed without any difficulties by placing the gel on a black background with naked eyes.

2.6 MALDI-TOF-MS

After detecting the *E. coli* cell total proteins in 2-D gels with EB-Mg stain or IZ stain, proteins were subjected to protease digestion and MS. Spots in 2-D gels were collected from three replicated gels to insure sufficient amount of sample available for MALDI-TOF MS analysis. For IZ stain, destain was performed by 2×8 min incubation in 1 mL 50 mM Tris buffer, 0.3 M glycine, pH 8.3 containing 30% ACN. While, for EB-Mg stain, the gel pieces were destained with 30% EtOH and 10% HAc for no less than 3 h. In-gel digestion and MS analysis were performed as described previously [24].

3 Results

3.1 Determination of optimal staining condition

3.1.1 Optimization of composition of staining solution

The optimal concentration of EB in staining solution was investigated ranging from 0.01 to 0.5% with certain amount of $MgCl_2$ in MeOH aqueous solution. The results showed that the 0.25% EB is appropriate as optimal concentration for detection considering high sensitivity and a good contrast. At the higher concentration of EB, the contrast

was poor, probably due to the fact that too much EB precipitated in the gel matrix leads the low amount protein bands to be obscure along with gel matrix.

As a smart point of this study, $MgCl_2$ is adopted in the staining solution, which can greatly improve both contrast and intensity of the protein bands. The optimal concentration of $MgCl_2$ was found to be 0.8% from 0.1–5%. In addition, to further investigate whether the introduction of inorganic salt might contribute to the increase in the staining sensitivity, various soluble inorganic salts were tested at a concentration ranging from 0.1 to 5%. As shown in Table 1, $MgCl_2$ was found much more sensitive than the remaining salts. In brief, the sensitivity of the staining was obtained for the following order: $MgCl_2 > Co(NO_3)_2 > K_2CrO_4 > Ni(NO_3)_2 > K_2Cr_2O_7$. EB-$K_2Cr_2O_7$ and EB-$Ni(NO_3)_2$ were less sensitive than EB-$MgCl_2$. Although EB-$Co(NO_3)_2$ and EB-K_2CrO_4-stained gels also provide slightly enhancing in sensitivity, a poor contrast was obtained by serious precipitation of EB. Furthermore, EB-K_2CrO_4-stained gels were not stable for long-term preservation caused by the serious decay on the sensitivity. Therefore, $MgCl_2$ was selected to be promising candidate.

For the determination of the optimal concentration of MeOH in staining solution, gels were stained by various concentrations of MeOH ranged from 25 to 60% in the fixed concentration of EB (0.25%) and $MgCl_2$ (0.8%) for 15 min. The results indicated that, intense staining occurred at 45% MeOH. In addition, it was reported that HAc plays an essential role in the formation of the protein-dye complex, of which the arginine, histidine, and lysine and tryptophan residues of a protein might bind electrostatically to carboxylic and phenolic groups of EB [23]. However, in this study, floc-like precipitation might be seriously produced, if any HAc is added into staining solution. Taking the advantage of HAc, it was introduced in the developing step of the gels after staining. Consequently, the constituent of staining solution was decided to be 0.25% EB, 0.8% $MgCl_2$ with 45% MeOH.

3.1.2 Determination of optimal staining time

After establishing the optimal composition of staining solution, all subsequent experiments were performed by incubating the gel in 0.25% EB, 0.8% $MgCl_2$ with 45% MeOH. As a next step, the optimal staining times were evaluated at 5, 10, 15, 20, 30, and 60 min. The results show that the 15-min staining results in the highest signal intensity for the protein amounts. For the maximum staining time, 60 min is sufficient for EB-Mg staining; however, the highest sensitivity was not obtained. Thus, 15 min staining is recommended.

Table 1. Screening of metal salts to optimize the sensitivity of EB

Staining performance	Metal salts				
	$MgCl_2$	$Co(NO_3)_2$	K_2CrO_4	$Ni(NO_3)_2$	$K_2Cr_2O_7$
Detection limit for SDS6H2	0.2 ng	0.2–0.4 ng	0.2–0.4 ng	0.4–2 ng	0.4–2 ng
Precipitation	No	Yes	No	Yes	No
Stability	Good	Good	No	No	Good
Contrast	Good	Good	Poor	General	Good

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

To determine the sensitivity of EB-Mg relatively to EB, EY, IZ, SYPRO ruby, and GA silver stains, a series of electrophoresis experiments were performed to compare the detection limit of each stain with molecular marker proteins (SDS6H2) by 1-DE. The results (shown in Fig. 3) indicate that among these methods the sensitivity of EB-Mg stain is approximately 0.2 ng of single protein band, similar to that of GA-silver stain, but higher than those of EB, EY, IZ and SYPRO Ruby stains of the same marker proteins. The sensitivity of EB-Mg was about two-folds higher compared with that of EY stain. Furthermore, total *E. coli* cell proteins were also separated by 1-DE and stained by a different method to thoroughly testify the above conclusion. As shown in Fig. 4, EB-Mg stain was also found to present a sensitive stain. In addition, the 2-D gels of total *E. coli* cell proteins stained with EB-Mg stain and IZ stain are shown in Fig. 5. Comparison of the spots visualized by the EB-Mg to those by IZ stain shows that most of the spots visualized by IZ stain are visualized by EB-Mg stain. Nevertheless, a better contrast and clearer 2-D map can be achieved by EB-Mg stain.

3.3 Linear dynamic range

The linear dynamic ranges were determined by comparing EB-Mg with IZ stains using molecular marker proteins (SDS6H2) of a serial dilution ranging from 0.1 to1000 ng. The results showed that the correlation coefficient of three types of proteins stained by EB-Mg were galactosidase (0.4–1000 ng, correlation coefficient 0.991), phosphorylase b (0.4–1000 ng, 0.989), and ovalbumin (0.4–1000 ng, 0.993). Although, the similar correlation coefficient was obtained by both methods, a relatively broader linear dynamic range for low detection limit was achieved by EB-Mg stain compared with that of IZ stain (shown in Fig. 6).

3.4 Compatibility with MALDI-TOF MS

Protein spots from 2-D gels visualized by EB-Mg and IZ stains were excised and digested under the same conditions with trypsin. Table 2 provides a summary of the MALDI data for all identified proteins of different staining methods. Comparative study on the MASCOT Score of both staining methods shows that 17 proteins stained with EB-Mg obtained higher scores than IZ, and 15 gained for IZ. Furthermore, for those protein

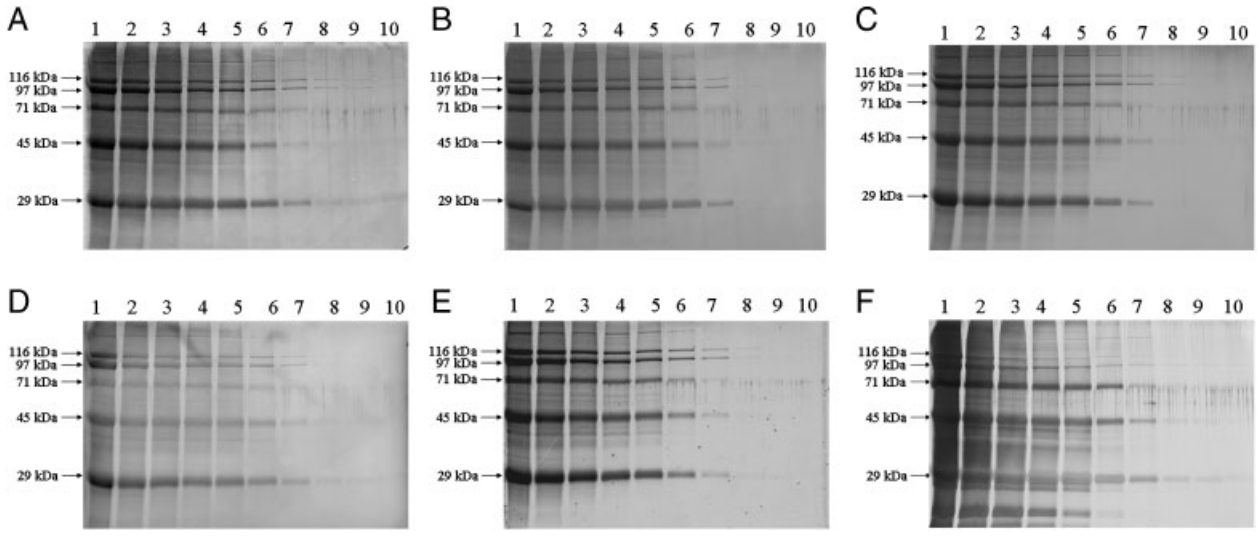

Figure 3. Comparison of the sensitivity of EB-Mg with other different detection methods in 1-D SDS-PAGE using standard marker proteins (SDS6H2).Serial dilutions of molecular weight standards-SDS6H2 in each wells (from left to right:lane 1, 000 ng/band; lane 2, 500 ng/band; lane 3, 250 ng/band; lane 4, 100 ng/band; lane 5, 50 ng/band; lane 6, 10 ng/band; lane 7, 2 ng/band; lane 8, 0.4 ng/band; lane 9,0.2 ng/band; lane 10, 0.1 ng/band) were separated on gels to compare the sensetivitise of different staining methods. After electrophoresis, gels were stained with (A) EB-Mg, (B) EB, (C) EY, (D) IZ, (E) SYPRO Ruby and (F) GA-silver stains.

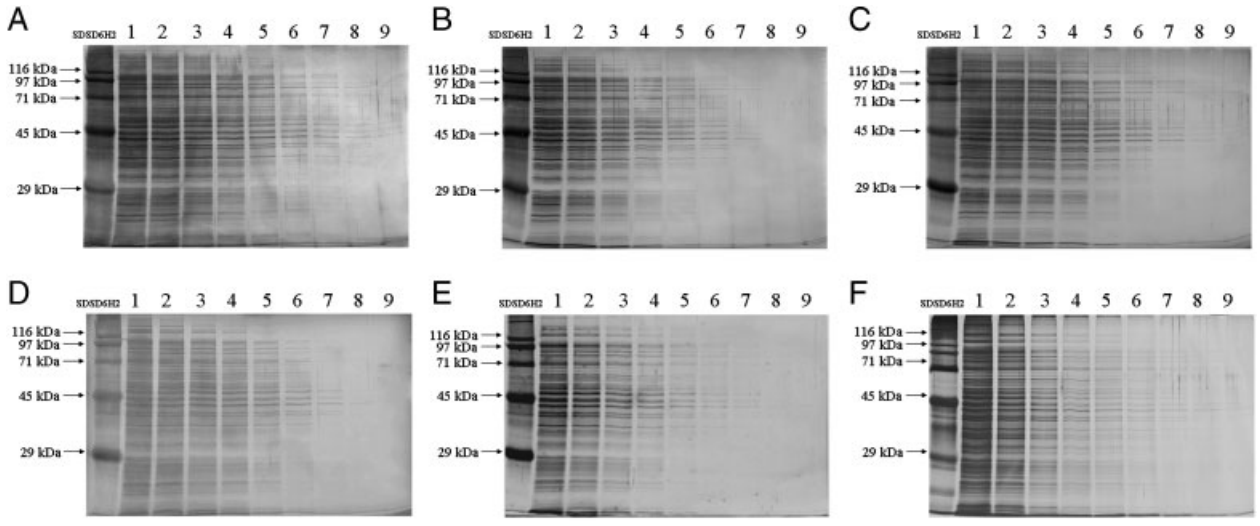

Figure 4. Comparison of the sensitivity of EB-Mg with other different detection methods in 1-D SDS-PAGE with total cell proteins of *E. coli*. Serial dilutions of lysis solution of *E. coli* in each wells (from left to right: lane 1, SDS6H2; lane 2, 1000 ng; lane 3, 500 ng; lane 4, 250 ng; lane 5, 125 ng; lane 6, 62.5 ng; lane 7, 31.3 ng; lane 8, 16 ng; lane 9, 8 ng; lane 10, 4 ng) were separated on gels to compare the sensetivitise of different staining methods. After electrophoresis, gels were stained with (A) EB-Mg, (B) EB, (C) EY, (D) IZ, (E) SYPRO Ruby and (F) GA-silver stains.

samples identified by both staining methods, 15 protein spots consistently resulted in more matching peptides when stained with EB-Mg and 13 protein spots consistently resulted in more matching peptides when stained with IZ. On the other hand, 4 protein spots gave similar numbers of matched peptide fragment notwithstanding the staining technique. According to these analyses, it can be concluded that the results of protein identification score and matching peptides show a similar trend for EB-Mg-stained protein samples comparing with those of IZ-stained protein samples.

4 Discussion

We describe a novel procedure for negative staining of proteins in polyacrylamide gels based on the interaction between EB and proteins in the presence of $MgCl_2$. The newly developed protocol is deemed as a further step for the study of negative detection method by using heterocyclic dye compared with the former published EY negative stain. In this study, $MgCl_2$ has a contribution effect on the solubility of protein–dye complex thus leading to great sensitivity improve-

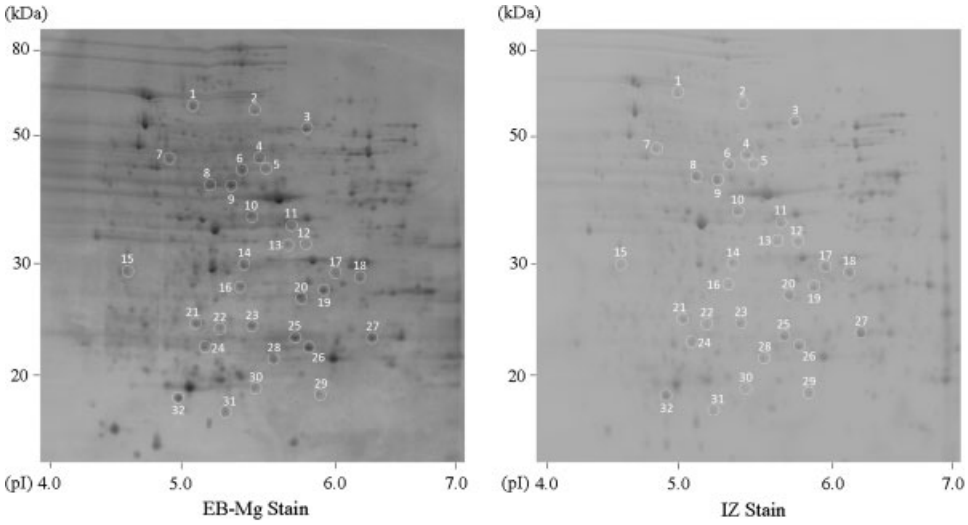

Figure 5. 2-D gels of *E. coli* protein sample stained with EB-Mg and IZ stains. The proteins were resolved in 4–7 linear pH gradient (IPG strips; 130 × 3 × 0.5 mm) and 12.5% SDS-PAGE (150 × 150 × 1 mm). Thirty-two representative spots were selected for further protein identification by in-gel trypsin digestion and MALDI-TOF MS.

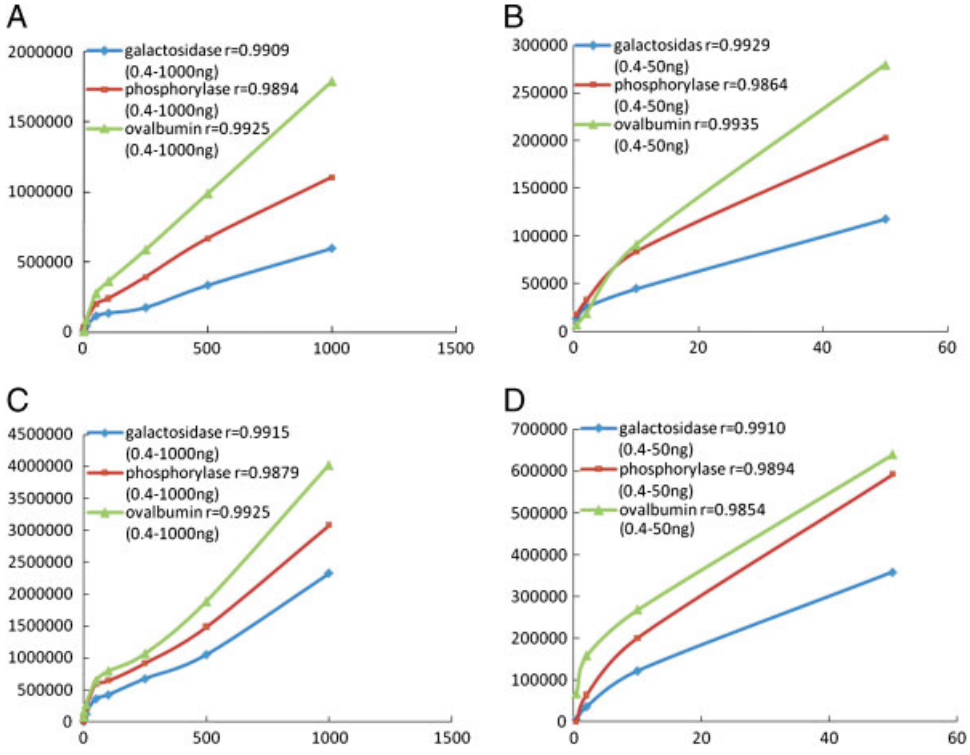

Figure 6. Comparison of the linear dynamic range using EB-Mg (A, B) and IZ (C, D) staining techniques. After staining, the protein bands of three representative proteins galactosidase, phosphorylase b and ovalbumin were estimated by TINA 2.09 image analyzing software program. (A) and (C) are graphs displaying the full concentration range evaluated (0.4–1000 ng) while (B) and (D) are graphs displaying the 0.4–50 ng range.

ment in measurement of the protein. Down to 0.2 ng of single protein can be detected within around 1 h. The main mechanism of EB-Mg stain is attributed that EB can interact non-covalently with proteins by means of both hydrophobic interaction between aromatic rings of EB and hydrophobic sites of the protein and by the electrostatic interaction between anionic carboxylic and phenolic groups of EB and the certain residues of protein to produce a stable water-soluble complex [19–23]. Then, at aqueous acetic condition, EB was converted into its neutral form and precipitated in the gel matrix to give an opaque background stain, while transparent protein bands/spots were achieved through the formation of water-soluble complex between EB and protein. On the other hand, protein solubility is governed by many factors, including pH, surface hydrophobicity, surface–charge distribution, size, salt-type, and salt concentration [25]. There are hydrophobic amino acids and hydrophilic amino acid in protein molecules. In aqueous solution, hydrophobic amino acids usually form protected hydrophobic areas while hydrophilic amino acids allow proteins to form hydrogen bonds with the surrounding water molecules [26]. If enough of the protein surface is hydrophilic, the protein can be dissolved in water. While,

Table 2. Comparison of *E. coli* proteins identified by MALDI-MS from EB-Mg and IZ-stained 2-D gels

Spot no.	Protein ID	Molecular weight	p*I*	MASCOT score		Peptides matched		Sequence coverage (%)	
				EB-Mg	IZ	EB-Mg	IZ	EB-Mg	IZ
1	Prolyl-tRNA synthetase *gi|157156008*	63 622	5.08	212	169	20	14	49	36
2	Bifunctional 2′,3′-cyclic nucleotide 2′-pHospHodiesterase/3′-nucleotidase periplasmic precursor protein *gi|215489563*	70 889	5.38	89	77	8	8	19	19
3	Bifunctional phosphoribosylaminoimidazole carboxamide formyltransferase/IMP cyclohydrolase *gi|218707625*	57 677	5.6	257	176	22	14	56	40
4	Seryl-tRNA synthetase *E. coli* O157:H7 str. Sakai *gi|15830232*	48 669	5.34	181	147	14	12	42	40
5	Peptidase PmbA *gi|157159292*	48 238	5.4	137	165	9	13	37	45
6	Chain A, crystal structure of *E. coli* argininosuccinate synthetase in complex with aspartate and citrulline *gi|17943194*	51 044	5.48	133	150	12	13	34	39
7	Phosphoribosylamine–glycine ligase *gi|157156020*	46 326	4.89	100	96	9	8	24	31
8	Isocitrate lyase *gi|556177*	47 485	5.44	177	216	16	17	47	54
9	Isocitrate dehydrogenase *gi|33383669*	43 192	5.33	280	198	21	15	61	50
10	Chain B, crystal structure of the complex of Adp and Mg^{2+} with dephosphorylated *gi|6980728*	41 338	5.36	134	119	13	12	42	40
11	Asparagine synthetase AsnA *gi|218702594*	36 804	5.38	66	77	5	6	26	33
12	NAD synthetase *gi|26247993*	30 805	5.3	134	137	11	11	45	53
13	6-Phosphofructokinase *gi|215489247*	35 176	5.48	71	114	5	9	35	54
14	Chain A, crystal structure of *E. coli* thioredoxin reductase refined at 2 Å resolution: implications for a large conformational change during catalysis *gi|157833918*	34 367	5.2	127	139	9	11	42	43
15	Aromatic-amino-acid aminotransferase *gi|237703616*	45 571	5.66	74	72	5	6	12	12
16	Phosphoglycerate kinase *gi|16130827*	41 264	5.08	75	67	7	6	19	19
17	Acetyl-CoA carboxylase carboxyltransferase subunit alpha *gi|15799867*	35 333	5.76	106	152	12	10	39	44
18	Chain A, structure analysis of prox in complex with glycine betaine *gi|46015450*	33 820	5.65	112	70	7	8	44	44
19	Chain A, X-ray structure of *E. coli* enoyl reductase with bound nad and benzo-diazaborine *gi|2914323*	27 943	5.58	111	78	12	8	42	43
20	2,3,4,5-Tetrahydropyridine-2,6-carboxylate *N*-succinyltransferase *gi|26246112*	30 001	5.56	116	88	12	7	43	30
21	Histidine ABC transporter, periplasmic histidine-binding protein *gi|194436225*	28 553	5.3	146	135	10	10	50	50
22	Site-specific recombinase, pHage integrase family *gi|168787584*	33 073	9.53	68	—	5		23	
23	Two-component response regulator *gi|15804972*	27 389	5.21	76	141	5	10	27	50
24	Lysine/arginine/ornithine transporter subunit *gi|16130245*	28 088	5.62	133	138	11	10	46	46
25	Adenylate kinase *gi|193064200*	25 741	6.14	71	143	10	13	51	59
26	Chain A, modular mutagenesis of a tim-barrel enzyme *gi|576304*	27 241	5.49	155	164	13	15	42	51
27	Chain A, *E.coli* cofactor-dependent phosphoglycerate mutase complexed with vanadate *gi|20149796*	28 408	5.86	152	181	11	14	50	56
28	Keto-hydroxyglutarate-aldolase/keto-deoxy-phosphogluconate aldolase *gi|15802263*	22 441	5.57	—	76		6		36
29	Chain A, the structure of clpp at 2.3 Å resolution suggests a model for ATP-dependent proteolysis *gi|3318853*	21 663	5.55	66	71	8	8	38	44
30	NADH dehydrogenase I, subunit nuoE *gi|397902*	18 849	5.4	74	64	6	5	49	37
31	Putative transferase *gi|253771897*	20 270	5.23	109	91	8	7	51	44
32	Inorganic pyrophosphatase *gi|15804817*	19 805	5.03	91	63	6	5	34	34

$MgCl_2$ is generally classified as a salting in and denaturation-inducing salt, it may salt in the proteins in the solvent, which contributes to the protein preferential hydration significantly [27, 28]. Furthermore, it is reported that low concentration $MgCl_2$ increases the protein solubility in aqueous solution, manifests the effect of $MgCl_2$ on its interaction with proteins [29, 30]. Therefore, it can be concluded that the character of $MgCl_2$ has great effect on the correlation between protein solubility and the preferential interaction of protein with dye that ultimately contributes to the increased staining sensitivity of EB dye. As a result, an increased intensity of protein signal was rendered by the presence of $MgCl_2$, which acting as a

sensitizer in the staining solution. However, more study is still needed to elucidate the mechanism of increased sensitivity contributed by $MgCl_2$.

On the other hand, as seen from Figs. 3 and 4, it can be observed that not all the high resolution staining of different proteins with different molecular weights was obtained by EB-Mg stain compared with other staining method. The differential resolution staining of proteins is primarily due to the different mechanisms by which each stain reacts with protein. For IZ, binding of Zn ions by the SDS–protein complex bands results in a differential distribution of free Zn ions along the gel. In the presence of imidazole, the free or weakly bound Zn ions are readily precipitated along the gel as zinc–imidazolate [31]. For SYPRO Ruby and silver, they both can interact with the basic amino acid in proteins to provide the visualization of protein [32, 33]. While binding of EB and EY to protein comes from hydrophobic and electrostatic interactions to form a stable water-soluble protein–dye complex. Overall, successful visualization of *E. coli* cell proteins in 1-D and 2-D with EB-Mg stain can be good demonstration for a wide applicability of this staining method, which makes it a high potential tool for proteomics studies.

The analytical method using EB-Mg not only has the advantageous analytical property of determining low amounts of proteins with accuracy and quantitation, it is also simple and rapid. In addition, the staining procedure is performed under normal conditions with commonly used reagents with good compatibility with analysis of MS, which makes it a good choice for routine use in protein research.

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